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SOME MINUTE MOVEMENTS IN PROTOPLASM

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In biological literature there are widely scattered records of apparently automatic movements of minute entities within the protoplasm of both unicellular and multicellular animals and plants; motions not referable to cyclosis nor to pedesis; having yet no name, accepted meaning or cause.

In the ciliated protozoa Folliculinidae, I studied these motions with the Zeiss immersion lens, in 1912, 1913 and again in 1945, 1946, and 1950, as well as in the related Stentoridae. Since these observations were scarcely mentioned in my papers (1923, 1945, 1946) I now bring them to attention, in the hope that others will find these organisms favorable for extended investigation with exact measurements and experiments to gain some better comprehension of such phenomena.

In folliculinids and stentors food is cast into the endosarc from the oesophagus to be carried in a food vacuole or "gastriole" slowly and individually through a long course, until finally undigested refuse is thrown out at the cytoproct. The transit of gastrioles is not in a visible tract nor in cyclosis, but each is individually handled as so often seen when yeast cells boiled and stained with Congo Red are taken as food, but not digested. Moreover, individual grains of yeast may be carried through the animal without large enveloping sacs, and the same is true of small linear crystals of the above stain.

The protoplasm of folliculinids and stentors is colored, commonly blue-green, and this color is concentrated chiefly in color bodies, "protrichocysts," scattered through the interior but crowded in lengthwise lines in the ectosarc. In *Stentor coeruleus,* where the interior is grossly vesiculated, these blue granules move about between vesicles as if gliding along films, but in clear homogeneous plasm they have no visible track. They go individually in various directions.

To overcome the difficulty of focusing in the thick mass the attenuated stalk of the fully extended animal is most favorable, as here the clear plasm forms a thin film but eight microns wide. In *Stentor igneus* individual red grains went in such stalks more than 15 microns at an estimated rate of 2¼ microns per second. All the interior was active with such movements resembling autos in a city going in diverse directions, stopping and starting. Some passed close to others going in opposite directions. In *Parafolliculina americana* Hadzi the trajectories of such particles were curved as well as straight. Their size was from ½ micron to 3 microns. In camera lucida sketches the particles magnified 2400 diameters went 10 to 100 times their own lengths in single trips at a rate of 2 to 3 microns per

second. The motions of the blue grains in *Stentor coeruleus* have been seen recently by Weisz (1949) who emphasizes the chemical structure of these bodies and concludes that they are mitochondria. That he saw the color bodies in the ectosarc constantly active in pedesis and going into the interior suggests that coverglass pressure may have liquefied the area observed. I considered the color bodies to be end products of digestion, of no use in metabolism; since in cannibal stentors and in blepharismas the ingested prey is digested except the color bodies which are gathered in a mass and cast out at the cytoproct. Moreover, when stentors stand long in one place color bodies are cast out and accumulate as a green mass about the feet; while in folliculinids lines of ectosarcal color bodies are cast out as part of the secretion that forms the temporary dwellings.

Some records of minute movements similar to these in the Folliculinidae and Stentoridae may be briefly enumerated as follows. Schultz (1863), who emphasized flowing as fundamental in living things, studied in detail motions in the fine anastomosing pseudopodial threads of Foraminifera which serve both for feeding and for locomotion. He saw that many granules acted independently, like people on a crowded sidewalk, swarming about amongst others, at times halting and trembling, but always at length going to an end of the pseudopod. Dead objects, grains of carmine, showed the same phenomena. Schneider (1906) also saw particles moved independent of flow in both Foraminifera and Radiolaria as well as in the stamen hairs of a common plant, the gourd, which proved a very favorable subject when Martin Heidenhain (1897, 1911) studied these cells with Zeiss water immersion and apochromatic oil immersion lenses. He saw individual particles moved along and concluded they were carried by invisible "minimum waves" of the surrounding plasm.

Somewhat as in forams, mycetozoa (myxomycetes) move and feed by means of protoplasmic networks within which currents of protoplasm are seen to carry innumerable colored granules; however, these currents reverse rhythmically, as so well demonstrated by Kamiya (1942). Loewy (1949), from facts discovered by Seifriz, concluded the cause of these flows was within them; every flow would be made up of minute individual acts; apparently erratic individual motions were due to temporary lack of agreement with the motions propelling the majority that made up the flow. This concept was independently arrived at by several investigators of that period.

That mitochondria also exhibit individual motions was demonstrated by the Lewises (1915) in tissue cultures of chick embryos.

Chromatophores, color-bearing cells of some animals capable of rapid color changes, also show both flow and individual motions of colored grains. Degner (1912) studied shrimp chromatophores and likened the movements of grains to those seen by Schultz in forams. The chromatophores of fish were studied by Ballowitz (1913) and in another species by Mathews (1931). In amphibia Haberlandt (1919) studied motions of the minute colored vesicles due to staining with intra-vitam stains. In reptiles chromatophores were studied by Schmidt (1917) in the scales of a lizard.

Additional instances of small motions are: those of nucleoli, seen only in certain stages of mitosis in Salvinia, by Hiraoka (1949); the movements of chondriosomes studied by Buvat (1953), in root cells of chickory; movements of gran-

ules in the erythrocytes of elasmobranchs, as demonstrated by Parpart (1950) by means of television microscopy; slight, but important, movements of chromosomes; and transport of chloroplasts in common green plants.

While the work that has been done on seeming automatic movements in protoplasm contains no extensive records of sizes and speeds, it seems that size varies much as in the folliculinids, while speed may be much greater. Observers fail to agree upon causes for such movements, but most all seek the causes in the plasm and regard the particles as passively moved by contraction, surface tension, sol-gel changes or a combination of chemical and physical activities.

Folliculinids and stentors, being complex animals of small size, offer advantages for observation and measurement and as various foreign bodies may be got into the protoplasm by the normal feeding mechanisms it should be possible to make comparisons between rates and courses of different inert particles, to aid in comprehending the forces involved in their propulsion. Are they propelled by forces acting only at the beginning of their trajectories or acting all along the lines of their courses? Experiments upon folliculinids might be used to test, or to confirm, some of the very interesting explanations of Professor William Seifriz (1953) arrived at in his recent work in Paris. He holds that the movements of particles, individually or in flow, are caused by action of the adjacent plasm, acting not only by contraction as in muscles, but also instigated by nerve-like foci in the plasm. Underlying these contractions, as well as contractions of muscles and activity of nerves, are inferred energy supplies from molecules having electric fields.

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MONOGENETIC TREMATODES OF GULF OF MEXICO FISHES. PART I. THE SUPERFAMILY GYRODACTYLOIDEA[1]

WILLIAM J. HARGIS, JR.

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The series of papers of which this is the first installment deals with the monogenetic trematodes which were recovered from 313 individual fishes representing 25 families. The hosts were collected during the period from September, 1951 to January, 1954. In all a total of 637 fishes of 98 species were examined throughout the study, but only 49 of these species carried the parasites to be reported in this series. When finally completed, a total of 75 species of Monogenea belonging to over 46 different genera will have been considered. The present paper presents data on several species belonging to the subfamilies Gyrodactylinae and Tetraonchinae.

While this study began essentially as a faunal survey and as such constitutes an entirely new locality record for all species, several aspects of perhaps greater importance have developed as it progressed. Some of these are: (1) an improvement of the taxonomy of the order, (2) the redescription of many previously described species, (3) an increased knowledge of monogenetic host-specificity, and (4) the discovery of several unusual structures, *e.g.* a giant cell in one species and what is thought to be a binucleated cell in another. These features will be treated in this and future installments.

The purpose of this work is to add to existing knowledge of the taxonomy and distribution of this very interesting and successful group of parasites. The Gulf of Mexico was chosen as the collection site because it represented an almost unexplored source of material.

Although the early workers, particularly European, have contributed much to our knowledge of the Monogenea by their pioneering research, the most useful and natural taxonomic schemes have been developed only recently. Most valuable of the recent studies have been those of Johnston and Tiegs (1922), Price's comprehensive series (1936 to 1943b) and Sproston's synopsis (1946). Price (1936 to 1943b), Sproston (1946) and Mizelle (1938) have treated the literature prior to 1946 so thoroughly that further comment is unnecessary. Since publication of Sproston's synopsis most of the 25 or 30 papers which have been published are species descriptions, locality records or morphological studies, but several of

[1] Contribution from the Oceanographic Institute and the Department of Zoology, Florida State University and the Biological Laboratories of the Citadel.

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The entire work is dedicated to Dr. Perry C. Holt and Mrs. Dolores M. Hargis.

broader scope have appeared. The most important of these are those of Brinkmann (1952), Palombi (1949) and Yamaguti (1953).

The taxonomic scheme employed in the present series is essentially that of Sproston (1946), who drew heavily on Price's works (1936 to 1943b). However, some emendations and additions have been made in the light of evidence which has been accumulated since 1946. Much of this evidence is derived from the recent contributions of Yamaguti (1942 and 1953) and studies of the present collection. An attempt has been made in the pages that follow to indicate by position or discussion the phylogenetic position of the families or subfamilies, but little has been done with the lower taxa because of present limitations of our knowledge. In dealing with the monogeneids it must be borne in mind that many of the existing systematic ideas are undoubtedly somewhat temporary. This is due to the dearth of information, embryological, faunal and morphological, concerning the order.

Materials and Methods

Collections were made at several points on the north and east coasts of the Gulf of Mexico. The sites are given with the discussion of each species. Hosts captured in waters adjacent to the Alligator Harbor Marine Laboratory of Florida State University were identified by the writer and Mr. E. B. Joseph. Those from distant sites were identified by the collectors.

Worms were prepared by the relaxation-fixation method of Hargis (1953). This technique has been found to yield good results as far as morphological detail, significant measurements and accurate ecological data are concerned and is especially suited for mass collections. Whole mounts were used exclusively, and where possible a large number of individuals were studied for comparison.

All measurements were made using an ocular micrometer and are cited in millimeters. In the case of curved structures measurements are of lines subtending the greatest arcs of those structures. In the descriptions given below the mean is given first, followed by the minima and maxima in parentheses. The number of measurements used to derive the mean is actually the same as the number of individuals measured; otherwise the actual number employed appears in parentheses before the measurements. All drawings were made with the aid of the camera lucida.

Taxonomic particulars, *i.e.* common names and authors, of the hosts are given with the description or discussion and are not repeated elsewhere.

Results

Order Monogenea Carus, 1863
Suborder Monopisthocotylea Odhner, 1912

Palombi (1949) is one recent author who apparently agrees with Fuhrmann's (1928) separation of this suborder into two suborders with different names, Monopisthodiscinea Fuhrmann, 1928 and Monopisthocotylinea (Ohdner, 1912), Fuhrmann, 1928, but until a more thorough evaluation of these groupings can be made, the writer prefers to follow the classification of Price (1937a), Sproston (1946) and Brinkmann (1952) and retain Odhner's groupings.

Superfamily Gyrodactyloidea Johnston and Tiegs, 1922
Family Gyrodactylidae Cobbold, 1864
Subfamily Gyrodactylinae Monticelli, 1892

It has been assumed by most workers that the members of the subfamily Gyrodactylinae, and perhaps also the Isancistrinae Fuhrmann, 1929, are more primitive than those of the other subfamilies of the superfamily Gyrodactyloidea. The writer would like to suggest that they may actually be more advanced. The reasons underlying this suggestion are: (1) both subfamilies are viviparous, usually considered an advancement, (2) both lack eyespots, usually suggestive of a long history of parasitic existence, (3) if elements of the haptor are really homologous as is presently suspected, it is just as possible that the dorsal anchors have been lost and their bars retained as it is that the evolution of these dorsal elements is just beginning with the bar appearing first, (4) the marginal hooks of *Gyrodactylus* are strikingly different from those of other gyrodactyloidids, apparently being more advanced. These possibilities should be investigated further. However, no change will be made in the relative position of Gyrodactylinae until additional evidence is available.

Genus *Gyrodactylus* Nordmann, 1832

Four species of this interesting genus were encountered on euryhaline fishes of the family Cyprinodontidae.

One of the difficulties encountered in morphological studies of members of the genus *Gyrodactylus* is their diminutive size. Another is the displacement and distortion of the internal organs caused by the uterine embryos. Both of these factors have combined to cause some uncertainty as to the precise identity of certain differentially staining structures and the proper terminology to be applied to others. For instance, some workers have applied the term "ootype" to a structure which is located at the posterior end of the uterus and often contains an ovumlike object. Because the ootype is usually considered to be that part of the oviduct where egg formation takes place it is possible that the name ootype is not applicable in this case since the worms are viviparous and may not form egg shells.

Gyrodactylus funduli n. sp.
(Figs. 1–3)

Host: *Fundulus similis* (Baird and Girard), Longnose Killifish, a euryhaline bentho-littoral cyprinodontid.

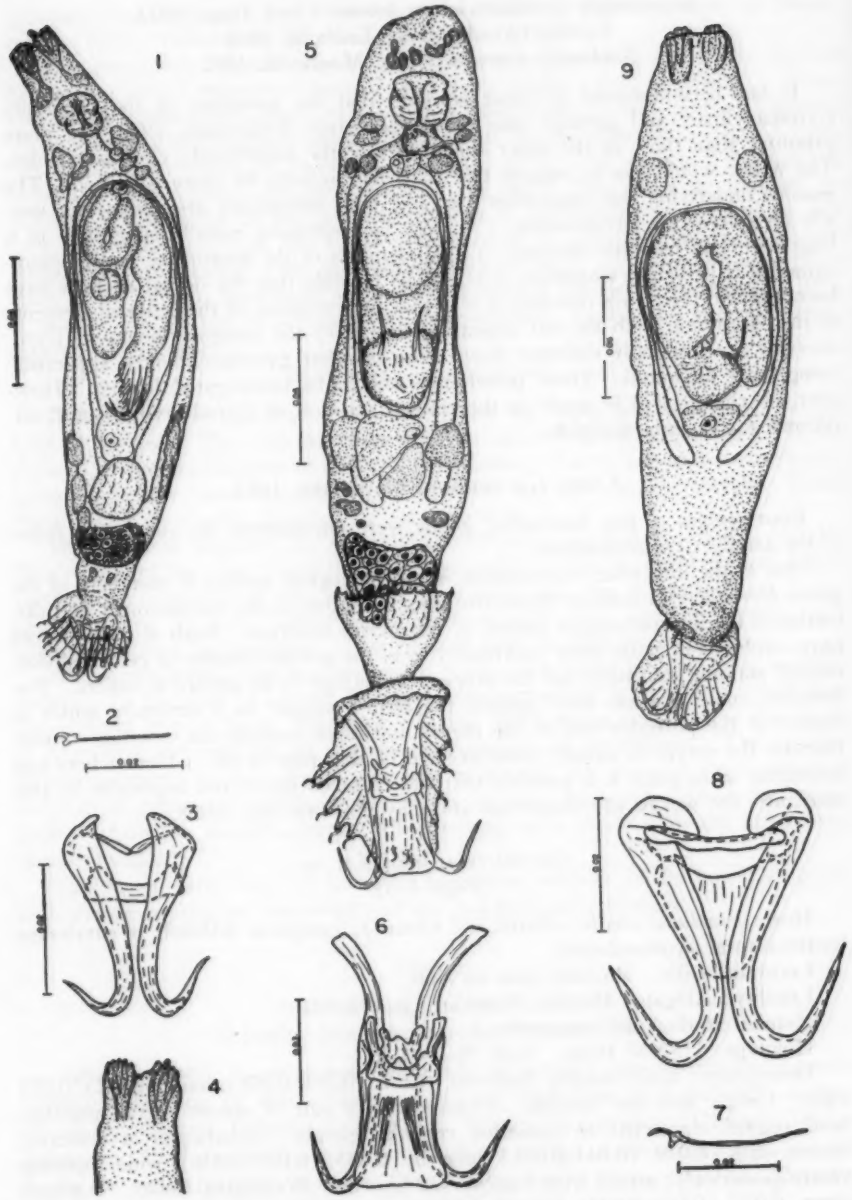
Location: Gills. Probably also on skin.

Locality: Alligator Harbor, Franklin Co., Florida.

Number studied and measured: 3.

Holotype: USNM Helm. Coll. No. 49330.

Description: Body weakly fusiform, 0.261 (0.255–0.268) long by (2) 0.051 wide. Cuticle thin and smooth. Prohaptor of a pair of antero-lateral, papillate head organs connected to posterior cephalic glands. Opisthaptor a concavo-convex disk, 0.049 (0.044–0.051) long by 0.035 (0.032–0.038) wide, opening ventro-posteriorly; armed with 2 anchors, 2 bars and 16 marginal hooks. Anchors stout, 0.032 (0.031–0.034) long by 0.008 wide, with short, angular, truncated



FIGURES 1–9.

roots, ventro-mesial knobs and strongly recurved tips. Ventral bar thick, (2) 0.012 (0.011–0.013) long by (2) 0.003 (0.002–0.004) wide, with anteriorly directed, truncate ends that articulate with the ventro-mesial knobs of the anchors. Dorsal bar delicate (2) 0.010 (0.008–0.012) long by (2) 0.001 wide, somewhat variable in shape, often appearing bipartite. Haptoral hooks (2) 0.021 (0.020–0.022) long, with long thin shafts and small, sickle-shaped tips. Pharynx bilobed, 0.019 (0.016–0.024) long by 0.014 (0.012–0.018) wide, strongly muscular; esophagus short. Gut bifurcate, crura unramified and not confluent posteriorly. Structure interpreted as testis roughly spherical, about (1) 0.024 in diameter, situated dorsally between posterior ends of intestinal crura. Structure interpreted as cirrus, globular, armed with a large curved spine and possibly smaller ones. Several darkly staining bodies laterally placed near the cirrus are not identifiable. Ovary elongate, somewhat bean-shaped, post-testicular. Structure interpreted as the "ootype" located immediately anterior to the testis. Vitellaria two irregular groups of darkly staining bodies lateral to the posterior ends of the intestinal crura. Large embryo *in utero*, (1) 0.090 long, and advanced enough to contain a second embryo.

Discussion: Though the closest relatives of *Gyrodactylus funduli* n. sp. are not apparent, it differs from all other known species of the genus in the following respects: (1) anchor roots obliquely truncate, (2) shape of ventral bar, (3) host.

Gyrodactylus prolongis n. sp.
(Figs. 4–7)

Synonym: *Gyrodactylus* sp. of Linton, 1940.

Host: *Fundulus grandis* Baird and Girard, Gulf Killifish, a euryhaline bentholittoral marine cyprinodontid.

Location: Gills. Probably also on skin.

Locality: Alligator Harbor, Franklin Co., Florida.

Previously reported host and locality: *Fundulus heteroclitus*, skin, at Woods Hole, Mass.

Number studied and measured: 3.

Holotype: USNM Helm. Coll. No. 49331.

Description: Body somewhat cylindrical, 0.348 (0.312–0.376) long by 0.076 (0.057–0.089) wide. Peduncle unusual, surrounded by an anteriorly directed skirt whose edge is armed by several cuticularized points; rest of cuticle thin and

Gyrodactylus funduli n. sp.

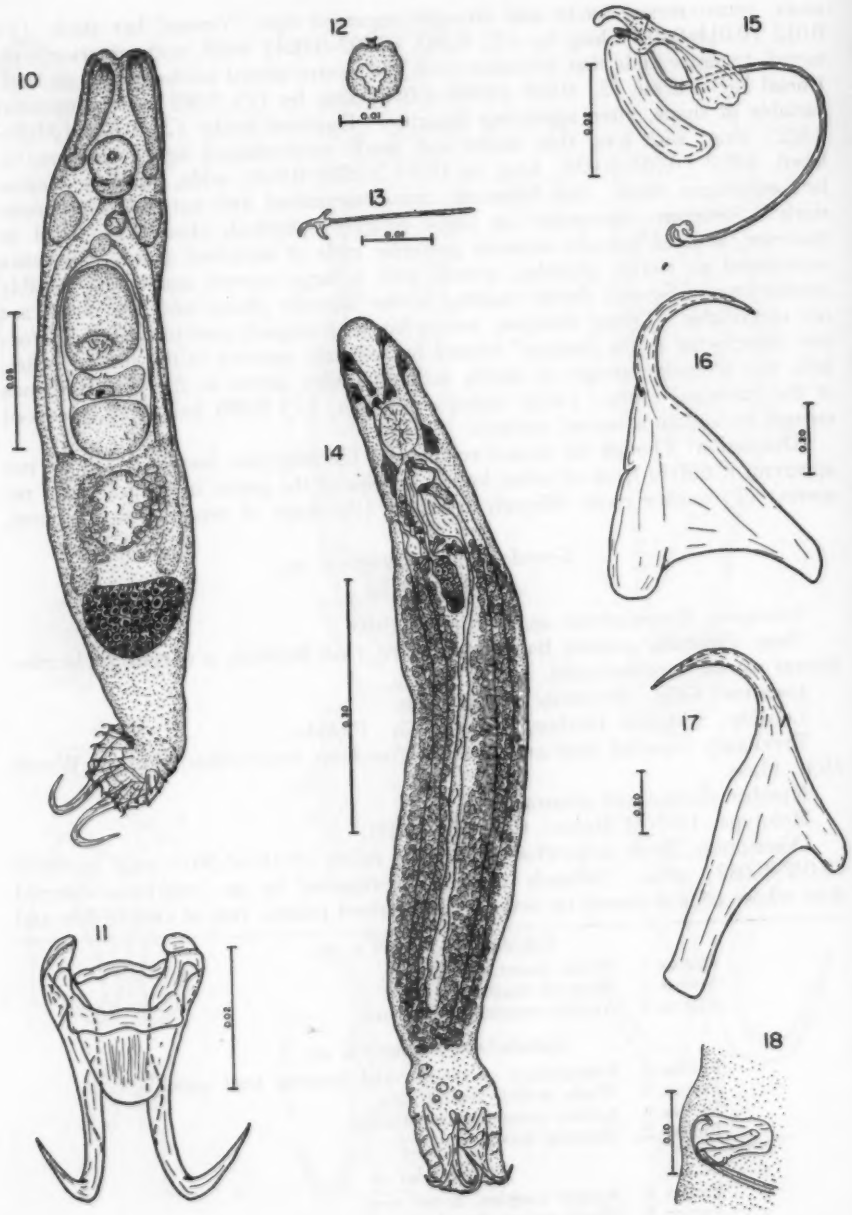
FIGURE 1. Whole mount, dorsal view.
FIGURE 2. Haptoral hook.
FIGURE 3. Anchor complex, dorsal view.

Gyrodactylus prolongis n. sp.

FIGURE 4. Enlargement of anterior end, showing head organs.
FIGURE 5. Whole mount, ventral view.
FIGURE 6. Anchor complex, ventral view.
FIGURE 7. Haptoral hook.

Gyrodactylus sp.

FIGURE 8. Anchor complex, dorsal view.
FIGURE 9. Whole mount, dorsal view.



FIGURES 10–18.

smooth. Prohaptor a pair of anterolateral papillate head organs, no cephalic glands observed. Opisthaptor irregularly oval to rectangular in shape, 0.081 (0.076–0.083) long by 0.049 (0.044–0.051) wide, armed with 2 anchors, 2 bars and 16 marginal hooks. Anchors relatively long, 0.075 (0.074–0.076) long by 0.010 wide, with exceptionally long roots, ventro-mesial knobs and strongly recurved tips. Ventral bar stout, 0.019 (0.018–0.023) long by 0.006 (0.005–0.007) wide, with long anteriorly projecting ends. Ventral shield rectangular, projecting posteriorly from ventral bar. Dorsal bar stout, butterfly shaped, 0.011 (0.009–0.014) long by 0.007 (0.005–0.008) wide. Haptoral hooks radially arranged, with long, thin shafts and sickle-shaped tips, (2) 0.041 long. Pharynx somewhat bilobed, strongly muscular, (1) 0.022 long by (1) 0.027 wide; esophagus short. Gut bifurcate, crura unramified and not confluent posteriorly. Testis ovoid, (1) 0.016 long by (1) 0.014, in posterior half of body; indication of vas deferens observed proceeding sinistrally from cirrus, suggesting vas deferens may course anteriorly on the left side of body. Cirrus muscular, spherical, 0.010 (0.009–0.012) in diameter, apparently armed with the usual hooks or spines. Unidentifiable darkly-staining bodies located near level of cirrus. Ovary irregular, partially dorsal to testis. "Ootype" subtriangular, appearing to contain an ovum, lying to right of midline between intestinal crura. Vitellaria consists of several irregular bodies near level of ootype. Embryo *in utero,* (1) 0.040 long by (1) 0.016 wide.

Discussion: This species was first reported by Linton (1940) but, apparently because of insufficient material, was not formally described. It differs from all other known species in the following respects: (1) unusual, skirt-like accessory holdfast on peduncle, (2) unusually long anchor roots, (3) rectangular shape of ventral shield.

The occurrence of this fluke on both *Fundulus heteroclitus,* Atlantic Ocean, and *F. grandis,* Gulf of Mexico, is probably a reflection of the close relationship of the hosts. This possibility is strengthened by the occurrence of *Gyrodactylus stephanus* Mueller, 1937 on both fishes.

Gyrodactylus sp.
(Figs. 8–9)

Host: *Cyprinodon variegatus* Lacépède, Variegated Minnow, a euryhaline bentho-littoral cyprinodontid.

Location: Gills. Probably also on skin.

Locality: Alligator Harbor, Franklin Co., Florida.

Gyrodactylus stephanus Mueller, 1937

FIGURE 10. Whole mount, dorsal view.
FIGURE 11. Anchor complex, ventral view.
FIGURE 12. Cirrus.
FIGURE 13. Haptoral hook.

Amphibdelloides narcine n. sp.

FIGURE 14. Whole mount, ventral view.
FIGURE 15. Cirrus complex.
FIGURE 16. Ventral anchor.
FIGURE 17. Dorsal anchor.
FIGURE 18. Vagina.

Number studied and measured: 1.

Slide in personal collection.

Description: Body weakly fusiform, 0.287 long by 0.064 wide. Prohaptor a pair of antero-lateral, papillate head organs; cephalic glands not seen. Cuticle thin and smooth. Opisthaptor oval, 0.044 long by 0.038 wide, concavo-convex, opening ventrally, armed by 2 anchors, 2 bars and 16 marginal hooks. Anchors stout, 0.040 long by 0.007 wide, with anteriorly recurving tips and characteristic long, mesially folded roots. Ventral bar larger than dorsal, 0.024 long by 0.005 wide, ends articulating with each anchor. Ventral shield subtriangular, projecting posteriorly from ventral bar. Dorsal bar slight, 0.023 long by 0.001 wide, somewhat irregular in outline and situated between the anterior ends of the anchors distally. Haptoral hooks 0.024 long with long, thin shafts and sickle-shaped tips. Pharynx not observed. Gut apparently bifurcate, crura unramified and not confluent posteriorly. Embryo *in utero* 0.094 long by 0.049 wide. Intensely staining body posterior to embryo may be a part of "ootype" because it appears to contain an ovum.

Discussion: Although this trematode is probably new to science, the present material is insufficient for formal description and naming. It differs from other *Gyrodactylus* spp. in the following respects: (1) length and abrupt medial curvature of anchor roots, (2) shape of ventral shield, (3) host.

Gyrodactylus stephanus Mueller, 1937
(Figs. 10–13)

Host: *Fundulus grandis* Baird and Girard, Gulf Killifish, a euryhaline, bentholittoral cyprinodontid.

Location: Gills. Probably also on skin.

Locality: Alligator Harbor, Florida.

Previously reported host and locality: *Fundulus heteroclitus* from marine habitats in the vicinity of Baltimore, Md.

Number studied: 6.

Number measured: 2

Homotype: USNM Helm. Coll. No. 49332.

Redescription: Body weakly fusiform, almost cylindrical, 0.328 (0.299–0.357) long by 0.044 (0.038–0.051) wide. Cuticle apparently thin and smooth. Prohaptor a pair of antero-lateral papillate head organs connected posteriorly to small cephalic glands. Opisthaptor oval, 0.048 (0.044–0.051) long by 0.038 (0.032–0.044) wide, concavo-convex, opening ventrally; armed with 2 anchors, 2 bars and 16 marginal hooks. Anchors stout, 0.042 (0.039–0.044) long by 0.007 wide, with ventro-mesial knobs and dorsally-directed wing-like expansions. Ventral bar stout, 0.020 (0.019–0.020) long by 0.004 wide, ends curved anteriorly and articulated with the ventral knobs of the anchors. Delicate ventral shield directed posteriorly from ventral bar. Dorsal bar somewhat irregular in outline, slight, 0.016 long by 0.001 wide. Pharynx bilobed, strongly muscular, 0.019 (0.018–0.020) long by 0.016 (0.012–0.019) wide; esophagus short. Gut bifurcate, crura unramified and apparently not confluent posteriorly. Testis ovoid, slightly postequatorial, 0.031 (0.028–0.034) long by 0.030 (0.028–0.031) wide, located between the posterior ends of the intestinal crura. Cirrus spherical,

muscular, 0.008 in diameter, armed with a clearly discernible spine, located dorsal to gut fork and slightly dextral to midline. Unidentified darkly-staining bodies placed lateral and posterior to the cirrus. Ovary somewhat irregular, post-testicular. Vitellaria apparently in confluent masses, probably also follicular, located antero-lateral to testis around intestinal crura. Ovoid structure immediately anterior to the testis appears to be a seminal receptacle because of presence of sperm. "Ootype" immediately anterior to receptacle. Embryo *in utero*, 0.040 long by 0.033 (0.032–0.034) wide.

Discussion: *G. stephanus* has been redescribed and figured because of the lack of detail in the original description.

As mentioned above, both *G. prolongis* n. sp. and *G. stephanus* occur on *Fundulus heteroclitus* and *F. grandis* and probably reflect the close relationship of these hosts. The latter species of fish is considered the Gulf counterpart of the former by some ichthyologists. Further study of the monogenetic trematodes infesting these euryhaline fish which have fresh-water relatives may prove interesting from ecological and zoogeographical standpoints.

Family Dactylogyridae Bychowsky, 1933
Subfamily Tetranochinae Monticelli, 1903

Tetraonchids are so small that, despite their usual large numbers, comparatively few workers have collected them. Well over a dozen species were taken during this study although only nine will be reported in the present series. The rest will be treated at some future date.

The systematics of this subfamily are in such an unsatisfactory state that a careful and thorough revision is indicated. Because of inadequacies of material and time this revision must be delayed; therefore, the species reported in this series are placed in available genera according to contemporary criteria in the hope that such action will facilitate the future sorting of species.

The chief reasons underlying these taxonomic difficulties appear to be the following. The characters used to separate many genera are not of sufficient importance to support such action. Also, the number of known species and genera has increased so rapidly that it has not been possible for systematists to keep abreast. Many of the recent workers who have made revisions in the subfamily have drawn their samples from either fresh-water or marine habitats, rarely both, and consequently have presented a somewhat distorted view; this is largely due to the current usage of different taxonomic criteria in the two ecological groups. The lack of a clear terminology also aids in obscuring details of importance, *e.g.* the term "accessory piece" is used by some to include both ornamentations of the cirrus itself and extra sclerotized structures located near the cirrus, others restrict this term to the latter, as it should be.

With the addition of the new genera of Yamaguti (1942 and 1953) and the recent reinstatement of *Haplocleidus* Mueller, 1937 by Hargis (1952) (Palombi, 1949, also recognized this genus) the number of tetraonchid genera stands at 24. This appears to be too many. In the light of new evidence it now appears that the taxonomic action by Hargis (1952) with regard to the genus *Haplocleidus* is not very significant because it is improbable that length and slight shape differences between the dorsal and ventral anchor pairs are of generic value. Later

work may show these groups to be subgeneric or infrageneric but they must be re-evaluated.

Some of the other characters which must also be reviewed are: (1) confluency or separation of the posterior ends of the intestinal crura; in actual practice this character is often difficult to detect because the gut is obscured by the vitellaria; (2) the side on which the vaginal pore opens; Brinkmann (1952) and others have contended that even complete absence of the vagina in monogenetic worms is not of much significance; (3) similarity or dissimilarity of bars; Yin and Sproston (1948) have remarked that bar dissimilarity may vary according to the opinion of the worker and is, therefore, not dependable as a taxonomic gauge. There are other difficulties but those listed will suffice to indicate the unsatisfactory state of affairs. The above statements may be easily verified by attempting to use the keys of Price (1937a) and Sproston (1946) and comparing their key generic characters with those of Yamaguti (1942 and 1953).

In the opinion of the present writer the taxonomy of Tetraonchinae will be considerably improved if, in addition to the evaluation recommended above, future workers make it a practice to give detailed figures of the entire worm being described and not just the taxonomic "hard parts." Too often characters of significance in the soft body parts and the general body shape are thus obscured. Also data concerning the hosts and their systematic or ecological relationships with each other may provide valuable systematic clues.

Genus *Amphibdelloides* Price, 1937

This genus is distinguished from its close relative *Amphibdella* Chatin, 1874, by the lack of lobes on the opisthaptor and the presence of a haptoral bar. The present writer suspects that these characters are not of generic importance and that later study will confirm this suspicion. Perhaps they are subgeneric in stature. However, Price's genus is retained pending further evidence.

Amphibdelloides narcine n. sp.
(Figs. 14–18)

Host: *Narcine brasiliensis* (Olfers), Torpedo Ray, a bentho-littoral marine torpedinid.

Location: Gills.

Locality: Alligator Harbor, Franklin Co., Florida.

Number studied: 274.

Number measured: 5.

Holotype: USNM Helm. Coll. No. 49333.

Paratype: USNM Helm. Coll. No. 49334.

Description: Body elongate, weakly fusiform, 0.903 (0.790–1.873) long by 0.161 (0.147–0.191) wide, rounded anteriorly, narrowed posteriorly to a wedge-shaped haptor. Cuticle thin and smooth. Prohaptor 2 paired clusters of about 3 small head organs each, connected posteriorly to cephalic glands. Opisthaptor wedge-shaped, rectangular in frontal view, 0.124 (0.108–0.146) long by 0.149 (0.102–0.248) wide; armed with 2 pairs of centrally placed anchors, 1 bar, 16 marginal hooks, and usually with several circular, cuticularized pieces variously

located. Anchors slightly dissimilar in shape but similar in size, shafts and tips curved; ventral anchors, 0.093 (0.085–0.099) long, with wide base, roots fused to form a triangle, differing from dorsal anchors in the completeness of root fusion and shorter superficial roots; dorsal anchors, 0.100 (0.093–0.108) long, base wide, roots more completely separated, superficial roots longer, situated lateral to the ventral anchors. Haptoral bar stout, 0.035 (0.026–0.051) long by 0.013 (0.009–0.018) wide, somewhat variable in shape, usually terminally expanded, apparently homologous to the ventral bar of other tetraonchids. Six pairs of haptoral hooks marginally located, (4) 0.008 (0.007–0.009) long, with bulbous shaft terminations and small sickle-shaped tips, one pair between roots of ventral anchors. Mouth mid-ventral, immediately anterior to pharynx; buccal tube short. Gut bifurcated, crura unramified, apparently not confluent posteriorly. Testis usually very long and narrow, (4) 0.396 (0.338–0.456) long by (4) 0.025 (0.019–0.030) wide, extending from near posterior ends of vitelline fields to a level just posterior to ovary; vas deferens sinistral to ovary, between intestinal crura, expanding anteriorly to form a seminal vesicle before narrowing to join cirrus. Cirrus a long, narrow, curved cuticular tube, 0.065 (0.054–0.070) long by 0.001 wide, with a flange-like proximal base; two accessory pieces, piece I, to right, a stout, slightly curved bar with parallel sides and variously sculptured anteriorly, (4) 0.027 (0.023–0.031) long by (4) 0.005 (0.004–0.005) wide; accessory piece II to left, (4) 0.019 (0.018–0.022) long by (1) 0.003 wide, widened posteriorly, constricted medially, anterior end in the shape of a hook with a hole in its proximal protuberance through which the cirrus passes. Prostate reservoir saccate, usually posterior to cirrus. Genital pore common, slightly dextral to midline near gut bifurcation. Ovary saccate, pretesticular at about one-third level of body; oviduct short. Ootype short; uterus a thin-walled tube to genital pore. Vagina dextromarginal, with an exterior cuticularized plate surrounding the vaginal pore, vaginal tube cuticularized, expanding mesially to join the seminal receptacle which narrows to join oviduct anterior to ovary. Mehlis' gland around oviduct immediately anterior to ovary. Vitellaria follicular, near intestinal crura, mostly posterior to ovary, terminating near posterior ends of crura, a few anterior follicles dorsal to right crus; transverse vitelloducts probably joining oviduct near level of Mehlis' gland. Eggs not observed. No eyespots.

Discussion: *Amphibdelloides narcine* n. sp. is very closely related to *A. maccallumi* (Johnston and Tiegs, 1922) Price, 1937 which was found to be parasitic on *Tetranarce occidentalis* (Storer), probably the natural host, and *Squalus acanthias* Linn., probably an "unnatural" host, at Woods Hole, Mass. (Recently Alexander, 1954, reported a form that he identified as *A. maccallumi* from the gills of *Torpedo californica* Ayers taken from Cortez Bank off the southern California coast.) A study of USNM Helm. Coll. slide No. 35701 (listed by Price 1937b, p. 153 as 25701) shows that *A. narcine* differs from *A. maccallumi* in the following respects: (1) body smaller, (2) shape of vaginal pore sclerite, (3) accessory pieces, although arranged in the same fashion, are differently sculptured and shaped, accessory piece I more simply sculptured, (4) anchor roots more deeply cleft, (5) host. Both worms are very similar morphologically, indicating that they are closely related and have, perhaps, differentiated only recently. In addition, this close relationship of the parasites probably reflects a corresponding

relationship between their hosts, *Narcine brasiliensis*, *Tetranarce occidentalis* and *Torpedo californica*.

The occurrence of *A. maccallumi* on *Squalus acanthias* Linn. is probably accidental or abnormal although nothing is known of the conditions under which the hosts were captured or held prior to autopsy by the original author, MacCallum (1916), who reported it as another species. It is strongly suspected that many of MacCallum's host records are not "natural" due to the sources of host material, *e.g.* the New York Aquarium and the New York fish market.

Summary and Conclusions

The present paper is at once the introduction to and the first installment of a projected series of papers which will include the data gathered during a recent study of the monogenetic trematodes recovered from 49 species of Gulf of Mexico fishes. The introduction given herein includes a discussion of the scope and important aspects of the entire work and a short discussion of the literature of the order. In addition, the present state of the taxonomy of the order Monogenea has been briefly considered and the conclusion reached that monogenetic systematics is relatively young and plastic and that much research remains to be done before it can be stabilized.

Only the first portion of the data concerning the superfamily Gyrodactyloidea has been presented herein. Two new, one previously described and one unknown species of the genus *Gyrodactylus* have been described and discussed. These are *G. funduli* n. sp., *G. prolongis* n. sp., *G. stephanus* Mueller, 1937 and *G.* sp., respectively. It has been suggested that members of this interesting genus may actually be phylogenetically more advanced and not more primitive than other gyrodactylids as has heretofore been commonly assumed.

The systematics of the subfamily Tetraonchinae has been considered and the conclusion reached that the entire subfamily and its taxonomic characters should be carefully reviewed in an effort to establish a more natural scheme. In addition, *Amphibdelloides narcine* n. sp., the first of several new tetraonchids discovered, has been described and its relationship with *A. macallumi* considered.

Part II will continue presentation of data on the subfamily Tetraonchinae of the superfamily Gyrodactyloidea.

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ON THE DEVELOPMENT OF ENUCLEATED TRITON EGGS WITH AN INJECTED BLASTULA NUCLEUS

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The experimental method of nuclear transplantation in amphibian eggs reported by Briggs and King (1952, 1953) and King and Briggs (1954a, 1954b) provides a promising new tool for the study of nuclear differentiation during development. They have shown that it is possible to activate and enucleate eggs of the grass frog, *Rana pipiens*, by pricking, and then to inject nuclei from late blastula, gastrula, or older cells into them. Following this treatment, development in the best cases was almost normal to the tadpole stage. Their results indicate that, in Rana at least, nuclei from undetermined blastula cells and chorda and medullary gastrula cells are able to replace the zygote nucleus in development. However, their results also indicate (King and Briggs, 1954b) that as the differentiation of endoderm cells proceeds through neurula and early tailbud stages, the nuclei of these cells become less and less capable of participating in total development. Nevertheless, the nuclei of determined gastrula cells are themselves undetermined and totipotent in developmental capacity since (p. 77, King and Briggs, 1954a), ". . . nuclei from the presumptive medullary plate are *not* limited to participitation in neural differentiation but may participate as well in mesodermal and endodermal differentiation. The same situation exists with respect to the chorda-mesoderm nuclei, which also participate in all types of differentiation."

Briggs and King's method of activating unfertilized eggs of *R. pipiens* was by pricking with a clean glass needle. This, although activating the eggs, was not sufficient to cause them to undergo normal parthenogenetic development beyond the early cleavage stage. This is in agreement with the initial observation by Bataillon (1910) and many later investigators. In the hands of Briggs and King, the enucleation of activated *R. pipiens* eggs following the method of Porter (1936) was almost 100% successful. Three separate checks were used in determining success in enucleation; they were: a) pricked and enucleated eggs failed to cleave or to form cleavage furrows of any kind, b) enucleated eggs fertilized by normal sperm developed without exception into androgenetic haploids as determined by nucleolar number and epidermal nuclear size measurements at tailbud stage 18, and c) Feulgen-stained sections of morula to gastrula stage embryos with injected blastula nuclei showed Feulgen-positive material in the exovate resulting from

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enucleation which was interpreted as the remains of egg nucleus chromatin. These latter cases also possessed diploid-size nuclei in the embryonic cells which the authors concluded were derived from the injected blastula nucleus.

Waddington and Pantelouris (1953) have attempted nuclear injection experiments on egg fragments of the newt, *Triturus* (*Triton*) *palmatus*. They obtained cleavage and partial blastulae; however, no cases advanced to gastrula or later developmental stages. They suggest that technical difficulties in handling the material might be responsible for their failure to obtain development beyond the blastula stage.

It seemed appropriate to attempt to repeat the experiments of Briggs and King, and Waddington and Pantelouris on whole eggs of *Triton palmatus* and *Triton alpestris*, and to include chromosome analyses of experimental eggs. These species are particularly suited for cytological study owing to the low number ($N = 12$) and large size of the chromosomes. Application of the smear technique given in the following description of methods enables one to make chromosome counts on Triton eggs at any time during development. Thus, one is not dependent upon nucleolar number and size measurements of interphase nuclei in older embryos for this critical criterion for determining success in enucleation and nuclear transplantation.

It should be pointed out at this time that although none of the cases in the following experiments developed beyond the early gastrula stage, the results in general outline support the observations reported by Briggs and King, and Waddington and Pantelouris. In early developmental stages at least, an injected blastula nucleus of Triton can replace the zygote nucleus during cleavage and blastulation; however, the totipotency of post-blastula nuclei remains in question.

Material and Methods

1. Induction of ovulation

Gravid females collected in nature were injected on two successive days with 50 International Units Choriongonadotropic Hormone (Ciba, No. 542) dissolved in 0.1 cc. 2X Niu-Twitty solution (Flickinger, 1949; Niu and Twitty, 1953). Three or four days following the first injection, the females generally began to deposit eggs. They were then opened and the unfertilized eggs in the oviducts were removed to dry Petri dishes; there they were kept moist by evaporation from a small piece of wet filter paper cemented to the inside of the cover. Usually from 30 to 70 eggs were obtained from a single female.

2. Activation of the egg by irradiated sperm

The eggs of Triton, unlike those of Rana, cannot be activated by pricking with a clean glass needle, nor has it been possible in 64 attempts to activate the eggs by the injection of blastula nuclei alone. Since the experiments required chromosome-free eggs as hosts for injected blastula nuclei, an activation technique other than that used by Briggs and King had to be used in these experiments. The most successful method involved x-irradiation of sperm at 50,000 roentgens (7.5 minutes irradiation at 31 KV through a beryllium window and a 0.1-mm.

aluminum filter at 7 cm.), which is the dosage recommended as optimal for inactivation of sperm chromatin by Rugh (1939). The sperm ducts were removed from freshly killed males, placed on filter paper moistened with Niu-Twitty solution, and transferred to cellophane-covered Columbia watch dishes. The sperm were irradiated while still in the sperm ducts. Fertilization was carried out in the usual manner by tearing open the sperm duct and applying a small drop of undiluted sperm to the animal surface of the "dry" eggs. After 15 minutes the eggs were flooded with sterile tap water and were allowed to stand for 5 minutes before being removed by watch-maker's forceps from the rapidly swelling jelly capsules. In experimental and control series, 60% to 90% fertilization was generally obtained. Although polyspermy normally occurs in Triton and very frequently multiple sperm entry points can be seen as dark spots on the surface of the egg, Fankhauser (1925, 1932) has shown that only one sperm nucleus participates in normal development; the remainder degenerate. When several sperm penetrate an enucleated egg fragment, cleavage is abnormal and such germs never advance beyond the blastula stage (Fankhauser, 1934a). In the present experiments, haploid chromosome counts were consistently obtained from blastulae resulting from the fertilization of normal eggs with irradiated sperm; thus, there was conclusive evidence that the irradiation treatment was adequate for the inactivation of sperm chromosomes without seriously reducing the fertilizing capacity of the sperm.

3. Enucleation of activated eggs

It was not possible to eliminate the egg chromosomes by any x-ray dosage which did not in addition greatly lower the viscosity of the egg cytoplasm. It was decided therefore that more reliable results with less damage to the eggs could be obtained by using the enucleation method for Triton described by Curry (1931, 1936). Eggs were fertilized by irradiated sperm and removed from the jelly capsules in the manner described above. The eggs were then placed in numbered depressions on a wax-coated Petri dish filled with sterile Niu-Twitty solution. Each egg within its membrane was then pricked by a fine glass needle in the center of the lightly colored "egg spot" which marks the location of the second maturation spindle of the egg nucleus (see Fig. 1A). By means of a flat-tipped mouth- or hand-pipette the cytoplasm and maturation spindle in the region of the "egg spot" were then sucked out through the puncture in the egg membrane (Fig. 1B). Enucleations were carried out in groups of eight to twelve eggs immediately after they had been removed from the jelly capsules, that is, within thirty minutes following fertilization. The effectiveness of this technique decreases rapidly thirty to forty minutes after fertilization, since, by this time the second maturation division is complete and the egg pronucleus migrates from a superficial position into deeper levels of the egg as has been described for the axolotl by Stauffer (1945, Fig. 1). In the present experiments, the enucleation technique of Curry was approximately fifty per cent successful as determined by chromosome counts made on blastulae resulting from eggs fertilized by normal sperm. About one-half of such eggs proved to be haploid and half were diploid; the latter group represent failures in the enucleation technique.

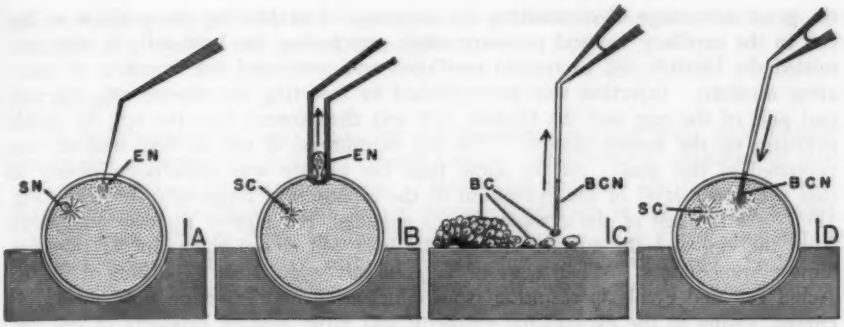

FIGURE 1. Diagram of transplantation technique. (A) Diagrammatic optical section of an egg fertilized with irradiated sperm (SN); the second maturation spindle (EN) of the egg is near the animal pole; a needle is shown pricking the "egg spot"; nuclei are shown greatly enlarged and are not visible in the living egg. (B) The same egg figured in 1A while the maturation spindle (EN) is being sucked out through the puncture in the egg membrane. The egg will then contain no active chromatin but the sperm centrosome (SC) may remain functional. (C) Donor blastula cells (BC) and the method of drawing one cell into the injection pipette are shown; note that the cell membrane of the blastula cell is not ruptured at the inner end and that the blastula cell nucleus (BCN) is visible as a small sphere during the injection procedure. (D) Injection of a blastula cell into an enucleated egg which then contains a diploid blastula cell nucleus (BCN) and a functional sperm aster (SC).

4. Injection of blastula nuclei

Eggs used in the injection experiments were fertilized with irradiated sperm and enucleated in the manner described above. The donor embryo consisted of a young normal diploid blastula (stage 7, Gläsner, 1925) which had been removed from its jelly capsule and egg membrane. It was placed in the operating dish along with the eggs to be enucleated. A small piece of the donor blastula was then removed by means of a hair or platinum loop (Baltzer, 1941). Always some of the cells along the edge of the excised piece detached from the fragment and these isolated cells were used for injection. Injections were performed immediately after enucleating a set of host eggs. Entire donor cells were used and no attempt was made to eliminate the cytoplasm. All injections were carried out free-hand by mouth-pipette. The tip of the injection pipette was a thin-walled capillary with an inside diameter of approximately one-third the diameter of the cell to be injected. Correctly constructed pipettes permitted a cell to be drawn into the lumen of the tube without breaking the cell membrane until almost all of the cell contents were contained within the tube. Very frequently the nucleus of the blastula cell could be clearly seen as a transparent sphere in the column of yolky cytoplasm drawn into the capillary tip (see Fig. 1C). The method of handling cells for injection involved first filling the pipette to the level of capillary equilibrium; then, a small amount of fluid was blown out just before a single blastula cell was picked up. The return flow of fluid into the pipette was used to draw the cell slowly into the capillary tip. The pressure exerted was gentle and constant and when the correct amount of fluid was first expelled, the cell contents remained as an undiluted mass at the tip of the pipette. This procedure had

the great advantage of eliminating the necessity of controlling the position of the cell in the capillary by oral pressure while puncturing the host cell; it also permitted the blastula cell to remain undiluted and prevented the injection of operating medium. Injection was accomplished by inserting the pipette into the animal pole of the egg and the blastula cell was then forced into the egg by gentle pressure on the mouth pipette. The cell membrane, if not already broken, was ruptured at this time. At the same time the pipette was withdrawn slowly so that the upper level of the cytoplasm in the pipette was constantly in view (Fig. 1D). As the last of the cytoplasm was expelled, the pipette tip was withdrawn from the egg and the surface coat of the host cell closed the point of puncture almost immediately. When eggs of *T. palmatus* were used, expulsion of the injected cell and cytolysis resulting from enucleation and injection only rarely occurred, owing to the exceptional elasticity and rapid healing property of the surface coat. Eggs of *T. alpestris* were not as satisfactory in this respect.

5. Cytological preparations

Chromosome analyses in the cases to be reported were made from semipermanent smear preparations of late cleavage and blastula stage embryos. The technique was a modification of that given by Darlington and La Cour (1947) for aceto-orcein smears of Drosophila salivary gland chromosomes. The stain consisted of 2.0 gm. orcein refluxed for four hours in 100 cc. 70% acetic acid; when cool, the stain was filtered and used full strength. Blastulae were divided along the animal-vegetal axis into two halves so that two smears could be obtained from a single embryo. A half-blastula in a drop of Niu-Twitty solution was placed on a clean dry cover glass and oriented so that the non-coated (inner) surface of the embryo was in contact with the glass. All excess fluid was removed and a large drop of alcohol-acetic fixative (three parts 100% ethyl alcohol and one part glacial acetic acid) was added directly to the tissue. After 10 to 15 seconds a second clean cover glass was placed on the fixed cells to flatten them and encourage their adhesion to the lower cover glass surface. After one minute, the preparation was flooded with alcohol-acetic fixative which freed the upper cover glass and permitted it to be slipped off leaving the flattened smear firmly attached as a single sheet to the lower cover glass. The preparation was then transferred to a Columbia cover glass staining jar containing additional fixative and was allowed to harden for two hours. For staining, the cover glass smears were placed flat in covered Petri dishes since aceto-orcein softens the tissue and the smears will detach and slip from the cover glass if held vertically. Smear preparations were stained in full strength aceto-orcein for 30 minutes; differentiated in alcohol-acetic fixative until the smear had bleached to a light pink (approximately 5 minutes); dehydrated in 100% ethyl alcohol, and mounted directly in euparal, or cleared in xylol and mounted in Canada balsam.

Following the above procedure, the individual cell membranes, although broken, remained sufficiently intact to permit the identification of single cell contents. Successful smear preparations have been made from two-, four-, and eight-cell stage eggs; however, in early stages such as these it was found advisable to wait for 30 to 60 seconds before adding the second cover glass to flatten the fixed cells in order that a degree of hardening could take place before the cell mem-

branes were ruptured. In this way it was possible to retain the identity of individual cells even at the earliest cleavage stages.

6. Controls

In each experiment, eggs from one female were used and they were divided among the experimental and two control groups. One control consisted of normal eggs fertilized by normal sperm. These cases provided the basis for determining per cent fertilization and normal tempo of cleavage in a given egg sample. Usually from 60% to 90% fertilization was obtained and at room temperature the eggs began first cleavage within five and one-half to six hours; successive divisions began at approximately one and one-half hour intervals. Experimental eggs usually lagged from one-half to two or three hours behind the normal eggs.

The second control consisted of normal eggs fertilized by irradiated sperm. These were used to determine the effectiveness of the radiation treatment in inactivating the sperm chromatin. The eggs were smeared and stained in the manner described above at the mid-blastula stage. Successful irradiations at 50,000 r consistently gave haploid embryos with all chromosomes of maternal origin. In these cases, minute chromatic particles were frequently observed in the smears and they are believed to represent fragments of sperm chromatin (see Fig. 2D). In series which were considered successfully inactivated by irradiation, none of these "sperm" fragments was of sufficient size to be confused with normal chromosomes. In general the larger "sperm" fragments were uneven in thickness and often appeared as club-shaped masses as shown in Figure 2D.

Experimental Results

A total of 14 experimental series was carried out in which inactivation of sperm by irradiation was considered successful. There were 278 experimental cases in all and, of these, 70 whole eggs were fixed in Zenker solution at various developmental periods (see Column I, Table I) and stored in 70% alcohol for later cytological examination. The general results from the experiments are summarized in Table I and, in addition, Table II gives in greater detail the observations obtained from Series 62 which was the best single experiment.

1. Types of cleavage

The 252 experimental cases which were not preserved before the onset of cleavage can be divided into the three groups given in Column II, Table I.

a. Eighty-nine failed to cleave as a result of failure in fertilization or from operative damage.

b. One hundred and eight underwent abnormal cleavage that resembled in variety and kind the cleavages reported by Fankhauser (1925, 1934b) for andromerogonic eggs. To this group belong Cases 12, 15, 18, 24, and 32 figured in Table II. As in the case of andromerogonic embryos, many such cases, although showing initial irregularities in cleavage, can develop into blastulae which externally appear to be normal (see Cases 12, 15, and 24, Table II). The study of sectioned embryos of this kind has not been done but one might expect that these irregularities may stem from accessory sperm asters which produced cells with-

TABLE I

Summary of results from injecting blastula nuclei into enucleated Triton eggs

Experimental series	Total cases	I Whole embryos fixed			II Cleavage			III External appearance of blastula			IV Chromosome counts on young blastula			V Chromosome counts on old blastula		
		Before cleavage	During cleavage	As blastulae	None	Clearly abnormal	Normal or nearly normal	Cytolyzed	Clearly abnormal	Normal or nearly normal	Haploid	Diploid or hyperdiploid	None	Haploid	Diploid or hyperdiploid	None
26	10	—	—	1	6	—	4	—	—	4	—	—	—	—	—	3
29	35	—	—	3	6	15	14	6	8	15	1	3	3	6	2?	5
30	15	—	—	—	10	4	1	—	3	2	1	1	3	—	—	—
35	14	—	—	5	2	9	3	2	5	5	—	—	—	1	—	4
36	10	—	—	—	3	6	1	1	3	3	2	—	3	—	—	1
37	12	—	—	—	6	3	3	1	3	2	1	1	3	—	—	—
38	12	—	—	—	5	5	2	—	3	4	—	—	—	2	—	5
43	31	—	—	—	8	15	8	—	9	14	11	3	9	—	—	—
44	10	—	—	—	3	6	1	—	3	4	—	—	—	—	—	7
48	30	18	9	—	3	5	4	—	—	—	—	—	—	—	—	—
55	9	—	—	2	1	6	2	—	3	5	1	1	4	—	—	—
56	9	—	3	—	4	4	1	—	—	2	2	—	—	—	—	—
62	32	—	3	—	18	12	2	1	4	6	3	7	—	—	—	—
64	49	8	11	7	14	18	9	—	6	10	4?	1?	4?	—	—	—
Total	278	26	26	18	89	108	55	11	50	76	22+4?	16+1?	25+4?	9	2?	25
Column Totals		70				163			126			72			36	

out chromatin (as described in andromerogons by Fankhauser, 1934b), or with non-functional irradiated sperm chromatin (as found by Briggs, Green and King, 1951).

c. Fifty-five, or approximately 30% of the experimental eggs which cleaved, developed normally. That is, they passed through normal or nearly normal two- and four-cell stages and gave rise to blastulae which were superficially normal or nearly normal in appearance (see Cases 6, 9, and 31, Table II). Aside from the previously mentioned delay in the beginning of cleavage, these cases maintained a nearly normal division rate.

2. Types of blastulae

Of the 126 cases which developed into partial or complete blastulae (Column III, Table I), 18 were preserved entire as is indicated in Column I, Table I. The remaining 108 cases were used in the preparation of chromosome smears.

The 36 cases listed in Column V, Table I, were permitted to develop until the normally fertilized control eggs had advanced to the late gastrula stage. None of the experimental eggs developed beyond the earliest crescent blastopore stage and most cases gave no indication of gastrulation at all. When these cases were opened, masses of loose cells were found filling the interior of the germ and it

TABLE II

Summary of results from experimental series 62

CASE NO.	CLEAVAGE			APPEARANCE OF BLASTULA (16–18 HOURS)		CHROMOSOME ANALYSIS	
	FIRST (5½–7½ HRS)	SECOND (7–9 HRS)	THIRD (9–16 HRS.)	EXTERNAL	BLASTOCOELE	FIGURES COUNTED	PLOIDY
6				NORMAL	NORMAL	7	HAPLOID
9			DELAYED	NORMAL	?	1	HAPLOID
24			DELAYED	NORMAL	NONE	2	HAPLOID
12				NORMAL	NONE	8	DIPLOID
15			DELAYED	NORMAL	NONE	8	DIPLOID
17			DELAYED	UNEQUAL CLEAVAGE	NONE	2	DIPLOID
7				HALF UNCLEAVED	NONE	11	HYPER-DIPLOID
18				UNEQUAL CLEAVAGE	NONE	9	HYPER-DIPLOID
31		DELAYED	DELAYED	NORMAL	REDUCED CAVITY	9	HYPER-DIPLOID
32		DELAYED		SOLID MORULA	NONE	2	HYPER-DIPLOID

See explanation in text.

was evident that developmental advance had stopped during blastulation as Waddington and Pantelouris (1953) also have reported.

In the present experiments ectodermal donor cells were usually used for injection; however, in 47 cases endodermal cells were used. No appreciable difference in results was noted when the two types of donor cells were employed and germs with transplanted endodermal nuclei developed as well as those with injected ectodermal nuclei. Unfortunately no positive data have been obtained which throw light on the question of nuclear determination in blastula cells, since none of the cases have developed far enough to show morphological or histological differentiation.

The 72 cases given in Column IV, Table I, were sacrificed for smear preparations between late cleavage and mid-blastula stage 7 while the individual cells still appeared to be quite healthy. However, when these embryos were opened, even those cases which externally appeared to be most nearly normal showed internal anomalies. The internal malformations ranged from a thickened animal hemisphere and reduced blastocoele to solid blastulae such as those given in Cases 7, 17, and 18, Table II in which the internal yolk mass was only partially cleaved or exhibited unequal rates of cleavage in different parts of the germ.

3. Chromosome analysis

Thirty-six cases were permitted to continue development in the hope that advanced embryonic stages could be obtained but none gastrulated. When chromosome smears were made from these embryos, very few chromosome counts could be obtained. Most of the nuclei were pycnotic and apparently mitotic activity for the most part had stopped during the latter half of the blastula period. In only nine cases could valid chromosome counts be obtained (Column V, Table I) and these were all haploid and undoubtedly represent cases in which the techniques of enucleation and injection had failed. Two questionable cases in Series 29 were found which had tangled masses of chromosomes which could not be counted but seemed to represent appreciably more than the haploid number; these are recorded in Column V, Table I, as questionable diploids.

The most instructive chromosome analyses were obtained from 72 cases sacrificed during late cleavage and early blastula stages. At this time division is still largely synchronous and many cases were presumably at interphase when smeared and presented no countable mitoses for study (see the 29 cases in Column IV, Table I). In the remaining 43 cases, mitoses were found and counts were made on all anaphase figures which were spread sufficiently to be interpreted. A very considerable aid in making the chromosome diagnoses was provided by the excellently detailed study of Triton chromosome morphology by Fankhauser (1934c). He has demonstrated that most of the chromosomes can be individually recognized by structural characters. The most readily identifiable of the twelve chromosomes of Triton is "Number 8" which is characterized by a near-terminal achromatic region on one arm which is attached to a small "satellite" chromatic region. Special attempts were made to identify the homologues of chromosome No. 8 as a supplement to the total chromosome count in determining ploidy in each germ. The results of the best experimental series, No. 62, are presented in some detail in Table II and examples of chromosome configurations obtained from smears of Cases 6, 18, and 7 are shown in Figures 2A, 2B, and 2C, respec-

tively. In these figures, chromosome No. 8 has been drawn in heavy lines to make it more readily distinguishable from the others. Column IV, Table I, gives a total summary of results on the 43 cases in which valid counts could be made. With this tabular material, it is unnecessary to give a detailed discussion of individual cases; however, it should be noted that the embryos fall into two major groups, namely:

Euhaploid (22 + 4? cases) which represent failures in technique.

Eudiploid or hyperdiploid mosaics (16 + 1? cases) which probably represent complete or partial success of the experimental method used.

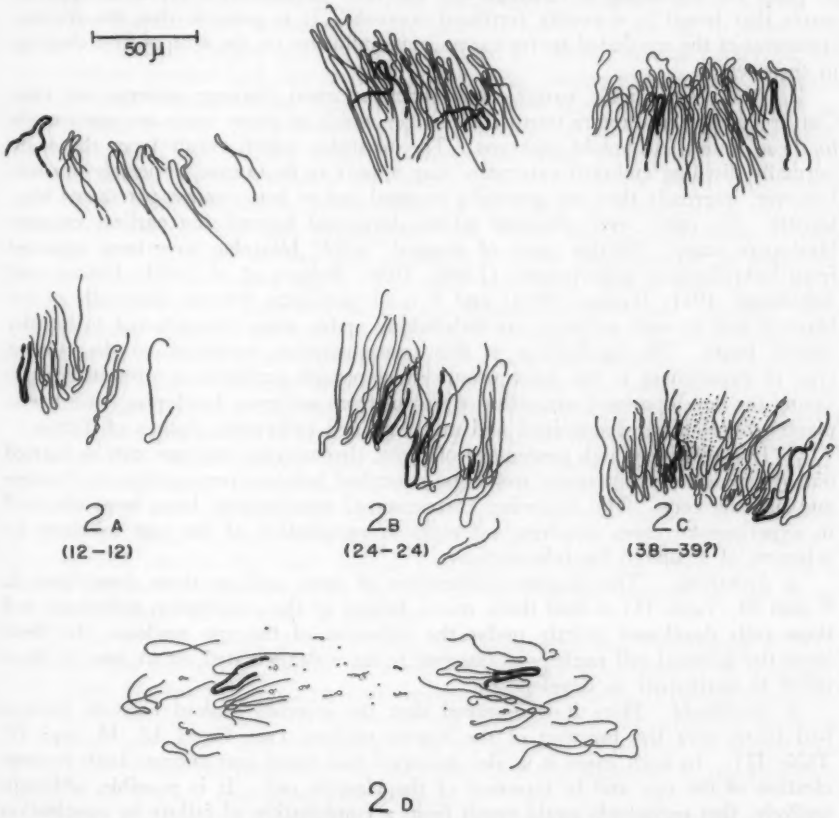

FIGURE 2. Camera lucida tracings of chromosome configurations obtained from aceto-orcein smears of Triton blastulae. (A) Euhaploid anaphase figure from Case 9, Series 62; Chromosome No. 8 (Fankhauser, 1934c) drawn in heavy lines. (B) Eudiploid anaphase figure from Case 18, Series 62; this case was a diploid-hyperdiploid mosaic. (C) Hyperdiploid anaphase figure from Case 7, Series 62; dotted lines in the lower half of the figure represent areas of crowding in which chromosome details are uncertain. (D) Euhaploid anaphase figure from a normal egg fertilized with irradiated sperm and showing an exceptionally large number of "sperm" fragments which are shown in outline; maternal chromosomes are shown in solid lines; See discussion in text.

Conclusions and Summary

The limited amount of data now available on nuclear injections in Triton does not permit one to draw definite conclusions concerning the developmental capacities and determination of injected blastula nuclei. However, several noteworthy facts can be pointed out which are derived from the data presented here.

1. Triton eggs can be enucleated, fertilized with sperm inactivated by irradiation at 50,000 r, and can develop under the influence of a blastula nucleus injected into the uncleaved egg cytoplasm. Some of these eggs will cleave and in almost all cases the beginning of cleavage and the time of successive divisions approximates that found in normally fertilized controls. It is possible that the division apparatus of the irradiated sperm exerts some influence on the time of first cleavage in these eggs.

2. Cleavage is highly variable and strictly normal cleavage patterns are rare. One frequently encounters unequal cleavages which in many ways are comparable to those found in haploid embryos. The blastulae which result from these abnormally dividing embryos externally may appear to be normal or nearly normal; however, internally they are generally atypical and at best possess a reduced blastocoele. No cases were obtained which developed beyond the earliest crescent blastopore stage. Similar types of stopped "solid" blastulae have been reported from hybridization experiments (Lüthi, 1938; Baltzer *et al.* 1939; Baltzer and Schömann, 1951; Baltzer, 1952) and it is of particular interest that cells of the blastula wall in such embryos are indefinitely viable when transplanted to healthy diploid hosts. The application of this transplantation technique to the current type of experiment is the most promising approach available at present for assaying the developmental capacities of tissues from embryos developing with transplanted nuclei from determined and undetermined embryonic tissues of Triton.

3. In a relatively high percentage of cases, chromosome analyses can be carried out by the aceto-orcein smear technique described herein when applied to cleavage and blastula cells. The following chromosomal constitutions have been obtained in experimental cases involving attempts at enucleation of the egg followed by injection of a diploid blastula nucleus:

a. Euhaploid. The simplest explanation of cases such as these (see Cases 6, 9, and 24, Table II) is that there was a failure of the enucleation technique and these cells developed strictly under the influence of the egg nucleus. In these cases the injected cell nucleus is assumed to have degenerated, or at least to have failed to participate in development.

b. Eudiploid. Here it is assumed that the injected diploid blastula nucleus had taken over the function of the zygote nucleus (see Cases 12, 15, and 17, Table II). In such cases it is also assumed that there was success both in enucleation of the egg and in injection of the blastula cell. It is possible, although unlikely, that eudiploids could result from a combination of failure in enucleation of the egg followed by suppression of the second maturation division with the result that all chromosomes would be of maternal origin. There is no way at present to determine with certainty that these diploid cases do indeed represent successful nuclear transplantations.

c. Hyperdiploid mosaics. These embryos possessed individual cells which contained different chromosome numbers that ranged from 24 to as many as 38

or more (see Cases 7, 18, 31, and 32, Table II). Mosaicism and heteroploidy cannot be adequately explained at present; however, it is possible that the maturation spindle of the egg was not removed or that some egg chromosomes remained in the egg following the attempt at enucleation of the host egg. If this had occurred and these chromosomes of maternal origin later became incorporated with those of the injected blastula cell, this could account for hyperdiploid mosaics. King and Briggs (1954a) have reported only one case of mosaicism in their experiments and it was haploid-diploid; however, they did encounter a rather high incidence of polyploidy and state (p. 77, 1954a) that "7 out of 17 embryos resulting from transfers of early gastrula nuclei were polyploid, while among the embryos derived from transfers of late gastrula nuclei, 13 out of 16 were polyploid." In their studies, ploidy was determined by nuclear size of epidermal cells and nuclear number and this method cannot yield precise information on heteroploidy; their results cannot, therefore, be compared directly with the results obtained with Triton. Nevertheless, their explanation of polyploidy in Rana might also apply to Triton. They suggest (Briggs and King, p. 458, 1952) that "The blastula nuclei are undoubtedly in various stages of mitosis when transplanted and there should be ample opportunity for doubling of chromosomes to occur before the nucleus has been transplanted and before the egg cytoplasm is ready to cleave." In order to test this possibility, smear preparations of individual Triton blastula cells were made. The cells were first sucked into an injection pipette in the manner described for the transplantation technique. Stained single cells revealed nuclei at all interphase and division stages and it seems quite probable that in different injected germs, partial or complete doubling of chromosome number could have occurred before the egg cytoplasm was prepared for division. It is therefore possible that the cases showing hyperdiploid counts may possess chromosomes that are all of donor origin. It is hoped that the study of preserved material fixed before cleavage may throw additional light on the behavior of injected blastula nuclei before the first cleavage of the host cell.

4. Under the conditions of these experiments and the techniques employed, there is no direct evidence to indicate that enucleated Triton eggs injected with a blastula cell can develop into normal post-gastrula embryos. Whether this is due to the lack of totipotency on the part of the injected nucleus or due to operative technique is not clear at present.

5. The present study, as well as that of Waddington and Pantelouris (1953), indicates that the eggs of Triton are a relatively unfavorable material as compared with Rana for nuclear injection experiments. The necessity of using irradiated sperm, the low percentage of successful egg enucleations, the irregular behavior of injected cell nuclei, and the cessation of development at the end of blastulation all tend to reduce the probability of obtaining clear-cut solutions to the problems attacked by nuclear transplant experiments.

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GROWTH OF OYSTERS WITH DAMAGED SHELL-EDGES

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During the past few years several papers have appeared dealing with the structure and other characteristics of oyster shells. Medcof (1944) discussed the structure, deposition and quality of the shell of the American oyster, *Crassostrea virginica*, while Korringa (1951a, 1951b) described at length the structure of the shell of the European oyster, *Ostrea edulis*, and also considered it as a habitat harboring numerous species of the epifauna of oysters. Korringa also incorporated in his papers an extensive review of the literature on shells of other oysters and related mollusks. Owen (1953) commented on the shells of lamellibranchs but confined himself almost exclusively to the matter of shell form.

No systematic observations, however, had been made to determine whether damage to oyster shells, such as breaking off their edges, would result in a more rapid growth, which would partly or wholly compensate for the loss caused by the breakage. The present studies were undertaken because we thought that information on this subject would be of both biological and practical significance.

A review of the literature shows that only the articles of Glaser (1903, 1905a, 1905b) and Nelson (1921) discussed a subject related to our problem. Glaser found that mis-shaped oysters, *Ostrea virginica* (now *Crassostrea virginica*), which had grown under unfavorable, overcrowded conditions, would eventually attain normal shape if replanted with ample room for unobstructed shell-growth. He also found that oysters, the growth of which had been inhibited by physical obstruction, would show rapid growth of shell in the affected areas after the obstruction was removed. Nelson (1921) later came to the same conclusions.

Breaking of shell-edges is common when heavy dredges are used for gathering oysters, as is the practice in Long Island Sound and in many other areas where the oyster beds are located at a considerable depth. The damage is especially severe if the oysters are dredged during periods when the new shell-growth is still thin and brittle. In Long Island Sound the most rapid increase in the length of oysters takes place during May, June and July, representing, respectively, 22.57, 19.03 and 22.12 per cent of the total annual increment (Loosanoff and Nomejko, 1949). The oysters dredged during that period usually have a considerable part of the new shell-growth broken off. Since in some areas the oysters may be handled several times annually, and because each time many of them may have the edges broken off, the accumulated loss of shell may be quite significant.

Our first experiment was begun July 18, 1946. Fifty oysters were divided at random into two equal groups. The oysters of the first group, designated as the control, were measured to determine their length, representing the greatest anterior-posterior dimension (Table I). The oysters of the second group were measured in a similar way, but after the initial measurements had been made the edges of their shells were filed off.

TABLE I

Original mean length and increase in mean length of shells of normal oysters and of oysters the shell-edges of which were filed off. Milford Harbor, 1946, 1947 and 1949. Measurements in millimeters

Group	Original length	Length after filing	Decrement due to filing	Length at end of season	Increase over		Per cent increase over	
					Original length	Filed length	Original length	Filed length
1946								
Control	69.5	—	—	91.7	22.2	—	31.9	—
Damaged	68.7	61.7	7.0	89.6	20.9	27.9	30.4	45.2
1947								
Control	92.7	—	—	102.9	10.2	—	11.0	—
Damaged	91.4	84.5	6.9	104.4	13.0	19.9	14.2	23.6
1949								
Control	85.4	—	—	112.8	27.4	—	32.1	—
Damaged	83.4	79.7	3.7	111.1	27.7	31.4	33.2	39.4

In filing, the shell material was removed all the way around the "bill" instead of only in one place, near its end (Fig. 1). The filing reduced the mean length of the shells by about 7.0 millimeters (Table I). The filed-off material consisted not only of recently-formed, thin and transparent shell but also of the thicker, old portion, some of which may have been formed the preceding year. After the filing the oysters were re-measured, and both groups were placed in wire trays, which were suspended side by side in Milford Harbor.

Since the filing was done by hand and slowly, the oysters had enough time to contract the edges of the mantle, avoiding injury to it. Because of this the amount of mantle tissue remained the same as before the filing, thus still possessing the original area of secretory surface for the formation of new shell.

For clarity of description, all the oysters, or groups of oysters, the shells of which were filed off in these experiments, will be called the damaged oysters, while the unfiled ones will be referred to as the control. The oysters used in these experiments were adults, three to six years old.

On December 6, 1946, when the water in Milford Harbor was already cold enough to stop shell-growth, the oysters were again examined. The measurements showed that during the period of the experiment the damaged oysters had added more new shell-growth than the controls. In general, since the date of filing, the mean length of the damaged oysters showed an increase of 27.9 mm., while that of the controls was only 22.2 mm.

The experiment was repeated again in the summer of 1947 using 50 individually-marked oysters for each group (Table I). The shells of one group were filed off on August 14, and then both the damaged and the control oysters were suspended together in the same tray in Milford Harbor. After the beginning of the hibernation period the oysters were again examined. It was found that, as indicated by the increase in length, the damaged oysters had not only added more

new shell-growth than the controls, but had actually outgrown them by the end of the season.

In 1947 both groups of oysters grew less than those used in 1946. This can be attributed to several considerations: first, the 1947 experiment was begun approximately one month later than that of 1946. Secondly, the oysters used in 1947 were considerably larger than those used in 1946 and their rate of growth could be expected to be slower than that of the smaller and younger oysters (Loosanoff, 1947). Finally, since the locations of the trays in which the oysters were suspended varied in the different years, the oysters may have been subjected to a somewhat different set of ecological conditions.

The experiments conducted in 1946 and 1947 showed conclusively that the oysters, the shell-edges of which had been filed off, compensated for this loss by forming new shell at a more rapid rate than the uninjured oysters living under identical conditions. However, since the experiments of these two years were not initiated at the beginning but approximately in the middle of the growing period, the experiments were repeated once more, starting in the spring when the oysters come out of hibernation, and continuing until late fall when the water again becomes too cold for oysters to grow.

On April 14, 1949, two groups, each composed of 110 individually-marked oysters, were treated as described above, and suspended in trays. Because the experiment commenced at the beginning of the shell-growing period, which in our waters usually occurs during the second half of April, the parts of the shells that were filed off consisted exclusively of old shell material that had been formed the year before. The restoration of that portion of the shell could be considered, therefore, as a true regenerative activity on the part of the oysters.

Final examination and measurements of the oysters were made on October 27. Three oysters were found dead in the control group but none in the damaged one.

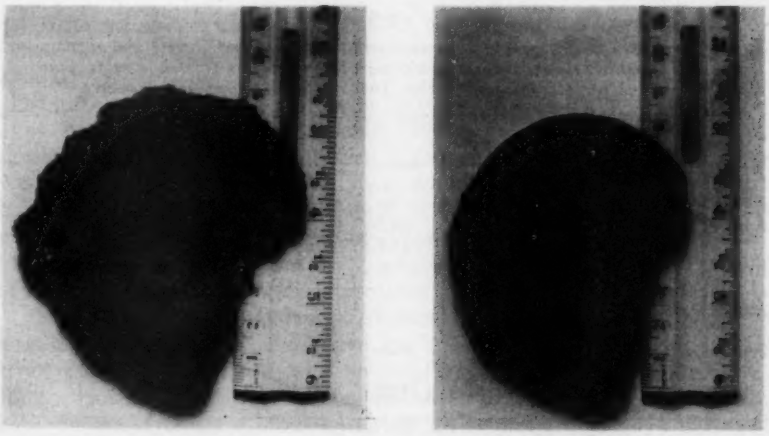

FIGURE 1. Photographs of the same oyster taken before and after the shell-edges were filed off.

The measurements showed that the damaged oysters again increased in length more than those of the control group (Table I).

Since the shell-edges of oysters may be broken off any time during the year, an experiment was devised to determine whether the tendency to compensate for such damages is present throughout the growing season, which extends from April to November (Loosanoff and Nomejko, 1949). The experiment was conducted as follows: On April 8, 1948 oysters that came from the same source were divided at random into five groups each containing 55 individuals, and the mean length of the oysters of each group was determined (Table II). The shells of Group 1, designated as the control, were not filed off during the entire period of observation. The shells of Group 2, however, were filed off on April 8, to provide information on the growth of oysters, the shells of which would be broken early in the spring, at the very beginning of the growing period. After filing, the oysters of Group 2 were again measured. All five groups were then suspended in wire trays in Milford Harbor, presumably under identical conditions.

As the season progressed, the oysters of Groups 3, 4 and 5 had their shells filed off. Each time measurements were taken before and after filing (Table II). Group 3 was filed off on July 13, Group 4 on August 9 and Group 5 on September 7. Thus, the entire season was roughly divided into four periods. Each time a group had its shells filed off and the length recorded, the length of the control group was also measured.

During this experiment 46 oysters were either lost overboard, died, or had their shells broken accidentally, thus becoming ineligible for further observations. The loss was especially serious in our control group when approximately half of it was lost near the end of the experiment. Nevertheless, 229 oysters were still available and our statistical analysis was based on these individuals. The number of oysters remaining in each group was as follows: Group 1—26; Group 2—54; Group 3—54; Group 4—51; and Group 5—44.

TABLE II

Original mean length of five groups of oysters and increase in mean length of normal oysters and of the oysters the shell-edges of which were filed off during different months of the growing period. Milford Harbor, 1948. Measurements in millimeters

Date	Groups				
	1	2	3	4	5
April 8, 1948	88.01	84.26* 81.70**	84.41	82.14	82.42
July 13, 1948	96.95	—	95.56* 90.68**	—	—
August 9, 1948	101.61	—	—	96.01* 91.79**	—
September 7, 1948	106.87	—	—	—	104.19* 99.26**
November 29, 1948	114.61	110.83	113.09	109.16	110.68

* Measurements before filing.
** Measurements after filing.

TABLE III

Decrements and increments of mean length of shells of normal oysters and of the oysters the shell-edges of which were filed off during different months of the growing period. Milford Harbor, 1948. Measurements in millimeters

Aspects studied	Groups				
	1	2	3	4	5
Decrement due to filing	—	2.56	4.88	4.22	4.93
Actual Length Increment (final length less initial length)	26.60	26.57	28.68	27.02	28.26
Per Cent Actual Length Increment	30.22	31.53	33.98	32.90	34.29
Total Length Increment (Actual Length Increment plus decrement due to filing of edges)	26.60	29.13	33.56	31.24	33.19
Per Cent Total Length Increment	30.22	34.57	39.76	38.03	40.27
Increase in length since filing, in millimeters	—	29.13	22.41	17.37	11.42
Corresponding increase of control, in millimeters	—	26.60	17.66	13.00	7.74
Per cent increase in length since filing	—	35.65	24.71	18.92	11.51
Corresponding per cent increase of control	—	30.22	18.22	12.79	7.24

Regardless of the supposedly random sorting of the oysters at the beginning of the experiment into five groups we found later that the control, Group 1, had a somewhat higher mean length than the other four groups (Table II). Analysis of variance showed that the differences in the initial length were significant beyond the .05 level. Therefore, to avoid in the analysis difficulties that would result from a disparity in the initial length, we decided to base the analysis of the amount of growth, shown by the different groups throughout the entire period of the experiment, not on the final length but on the difference between the initial and final lengths. This difference was termed the Actual Length Increment (Table III).

The analysis of variance, to test for homogeneity of the five groups on the basis of their Actual Length Increments, showed no significant difference among the groups. The conclusion was formed, therefore, that by the end of the growing period the damaged oysters showed approximately the same length increment as would have been expected of them if their shells had not been filed off. It should be remembered, however, that the Actual Length Increment does not include the increment needed to make up for the loss in length due to filing of the shells. If these two increments were combined to form the Total Length Increment (Table III), this would clearly show that all the damaged groups of oysters added more new shell and consequently must have increased in length faster during certain intervals than the control.

The stimulation caused by filing of the shells did not induce a significant increase in length beyond that of the oysters in the control group. The observations showed that, regardless of when during the growing period the edges of the shells

were filed off, the oysters grew just enough to compensate for this loss and then added to it the normally expected length increment.

Another measure considered in connection with our data was the Total Length Increment which, as already mentioned, consisted of the Actual Length Increment plus the decrement due to filing of the shells (Table III). (For the control group, obviously, the Actual Length Increment and the Total Length Increment were identical.) The analysis of variance for the four damaged groups on the Total Length Increment indicated that there were significant differences among the groups. This was especially true for Group 2 which showed a smaller Total Length Increment than the other damaged groups. However, this was to be expected because the decrement due to filing varied from group to group. Thus, the oysters of Group 2, which were filed earliest in the growth period, had the smallest decrement and, therefore, had to grow less in total length to achieve the same final length as the oysters of the other groups (Tables II and III).

It was thought that there might be a definite relationship between the amount of shell removed and the final length reached. However, it was established that the amount of damage, within our experimental scope, was not an important factor, that is, regardless of how great or slight the damage was, the individual oysters appeared to be stimulated only to the extent of compensating for the loss in length and then adding whatever additional increment would be expected under normal conditions. In other words, by breaking off more shell along the edges, the oysters could not be induced to reach proportionately greater length by the end of the growing season.

Although the experiments showed that breaking of shell-edges induces oysters to grow faster in length, we had no information concerning the rate at which such an increase proceeded. Because we had made no measurements on the oysters of Groups 2–4 after their shells had been filed, except the final one at the end of the growing period, there were no data for ascertaining the shape of the growth curve after the damage to the shells. It was thought, nevertheless, that since the damaged oysters showed practically the same Actual Length Increment as the control, the stimulating effect of shell-damage did not induce the oysters to grow at an accelerated rate throughout the remainder of the growing period. This was well illustrated by the oysters of Group 2, the shells of which were injured at the beginning of the growth period. This group, subsequently, had approximately eight months in which to grow at the accelerated rate if such rate were a reality. Nevertheless, the Actual Length Increment of these oysters not only did not exceed those of the three other damaged groups, but their Total Length Increment was the smallest (Table III).

Theoretically, there were at least two hypotheses regarding the rate of growth of oysters after damage to their shells. First, it could be assumed that following shell-damage the growth would continue at a somewhat accelerated but, nevertheless, even rate throughout the entire remaining portion of the growing period. The second possibility was that immediately after the damage the growth would proceed at a rapid rate but then, after sufficient growth had been added to the shell-edges to compensate for the original loss, the growth would decrease to the normal rate corresponding to that of the undamaged oysters. We thought the latter hypothesis was more probable because Group 5, which was damaged late

in the growing period, still came up to parity with the other groups in the length increment, thus indicating very rapid growth within a short period. However, to solve this matter definitely we carried on the following experiment:

Two groups of oysters were selected from a common source on May 21, 1954. The average length and width of the oysters of both groups were identical, being 80.6 and 64.5 mm., respectively. After that the shells of one group were filed off, reducing the average length to 77.0 mm. and average width to 60.0 mm. Both groups were then kept under the same conditions. A week later the average length and width of the control oysters were 81.0 and 65.0 mm., respectively, while similar dimensions of the damaged oysters were 79.5 and 62.5 mm. After two weeks the length and width of both groups became identical, being 82.0 and 66.5 mm., respectively, thus indicating that during this short period the oysters with filed shells had caught up with the control oysters. From then on the oysters of the two groups grew at approximately the same rate, showing no significant differences. This experiment has proven, therefore, that the second hypothesis was correct, namely, that in injured oysters the growth proceeds at a rapid rate for a brief period immediately after damage and then decreases.

The rapid growth of the new shell, representing "repair" or regeneration of the portion of the old one removed by filing, is probably due chiefly to the fact that the mantle of the oyster can now protrude farther along the edges of the shell. Because of this the secretory activities of the mantle involved in the formation of the edges of the shell are accelerated. The accelerated growth continues until the shell has grown enough to establish the normal ratio between the size of the oyster body and the length and width of the shell. After that the increase in length continues at approximately the same rate as in the undamaged oysters.

A rapid "repair" of the edges of shells is possible only if the oyster itself, especially its mantle, remains uninjured. If the mantle is injured, the oysters may not be able to show any increase in length until they recover. We have observed a number of such cases under laboratory conditions and also in nature. The best example of the latter was the condition of the Long Island Sound oysters following the extremely severe and prolonged storm of November, 1950. During this storm the oysters were rolled on the bottom by the wave action for many hours and with such force that the shell surfaces acquired almost a polished appearance, while the edges of the shells were rubbed off to such an extent that often the soft parts of the oysters protruded between them. Many oysters died because of injuries, which usually involved the mantle tissue. Those that survived did not resume normal growth but developed into stunted oysters with thick, irregular shells. Oysters of this type are still found in Long Island Sound four years after the storm.

The observations discussed in this paper are concerned almost exclusively with growth of shells as indicated by length measurements. They do not cover other aspects of growth and we know that it is possible that while in some of our experiments the new, thin shell-growth indicated an increase in length, the weight and volume of the oysters may have been smaller than before the shells were filed. Nevertheless, our auxiliary experiments, designed to ascertain the rate of growth immediately after filing, showed that after the edges of the shells had been filed, as shown in Figure 1, the growth in width proceeded at the same rate as the growth in length. This suggests that breakage of shell-edges is repaired by growth of

the same pattern regardless whether it is length or width that is involved in the damage.

Our studies represent only initial steps toward understanding various aspects of the growth of damaged oyster shells. The next experiment, which suggests itself, should consist of studying the growth of oysters, the shell-edges of which were broken several times during the growing period. The growth curve of repeatedly damaged oysters should differ considerably from that of normal oysters, or of oysters the shell-edges of which were broken only once during the season.

Another aspect of these studies could be directed toward ascertaining whether the growth of oysters of different ages and sizes, damaged in the same manner, would differ significantly from that of the oysters on which the present conclusions are based. Because we already know that during the first years the increases in length, width and depth of an oyster proceed at different rates than later in life (Loosanoff, 1947), we may assume such a difference to exist. This assumption would be in agreement with the observations of Glaser (1905b) who found that the recuperative power of overcrowded, mis-shaped oysters varies with age, young individuals recovering their normal shape more rapidly than old ones.

We wish to extend our sincere thanks to Barbara J. Myers for the statistical treatment of the data used in this article and to Miss Rita Riccio for her help in preparing and editing the manuscript. Our thanks are also due to several friends, especially Drs. T. C. Nelson and J. C. Medcof, who offered suggestions and advice.

Summary

1. These experiments, based on length measurements, showed that oysters, the shell-edges of which were broken off, compensate for this loss by forming new shell at a more rapid rate than undamaged oysters living under identical conditions.

2. Damage to shells does not stimulate oysters to grow at an accelerated rate throughout the remainder of the growing period. Immediately after the damage they grow rapidly to compensate for the loss and then continue to grow at the usual rate to add to their length the normally expected annual increment. Thus, no relationship was found between the amount of shell removed and the final length reached.

3. The ability of oysters to repair broken shell-edges and still grow to about the same length as undamaged oysters remains the same regardless of when during the growing season (April 8–September 7) the shells are broken.

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THE DEMONSTRATION OF TWO CHROMATOPHOROTROPICALLY ACTIVE SUBSTANCES IN THE LAND ISOPOD, TRACHELIPUS RATHKEI

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The remarkable ability of decapod Crustacea to undergo adaptive and rhythmic color changes has been under critical study for many years. Early investigators were concerned primarily with the structure of the chromatophores and with the possibility of neural control of pigment migration. Koller (1925), working with *Crago*, gave the first clear cut evidence for humoral control of the chromatophores. Perkins (1928) with *Palaemonetes* and Koller (1928) with *Crago* determined that the substance responsible for these effects was elaborated by some tissue of the eyestalk. Hanstrom (1937) successfully demonstrated that the observed chromatophorotropic activity of the eyestalk was localized in the region of the sinus gland. The extirpation experiments of Brown and Cunningham (1939) and Brown, Ederstrom and Scudamore (1939) presented conclusive evidence of the role of the sinus gland as a source of chromatophorotropins. With the exception of *Crago*, the decapods studied would seem to fall into one of two main categories: (1) those resembling *Palaemonetes* in that sinus gland extracts exert a concentrating influence upon the predominant pigment type and (2) those resembling *Uca* in that extracts of the sinus gland produce a dispersion of the dark pigment.

The presence of chromatophorotropins within the central nervous system was first shown by Brown (1933) for *Palaemonetes*. Subsequent work has established the presence of chromatophore activators within the central nervous system of most decapods. These factors have been found in some cases to have the same action as the hormones of the sinus gland, and in other instances they act antagonistically to them (Knowles, 1939; Brown and Ederstrom, 1940; Brown and Wulff, 1941; Brown, 1950; Sandeen, 1950; Enami, 1951; Brown, Webb and Sandeen, 1952).

The ability of isopod Crustacea to exhibit color responses has long been established and yet the exact nature of the mechanisms controlling these responses has not been clearly demonstrated. The presence of two hormones, one producing a dispersion of the melanin and the other a concentration of the melanin, was indicated in *Ligia oceanica* by the unique experiments of Smith (1938). However, Okay (1945) working with *Idothea baltica* and *Sphaeroma serratum* reported that the responses observed were dependent upon the presence of a single hormone.

Studies on the effects of extracts of isopod heads have yielded apparently conflicting results. In general, extracts prepared from isopod heads when injected into isopods of the same or related species have produced a concentration of the melanin (Kleinholz, 1937; Okay, 1945; Suneson, 1947; Carstam and Suneson, 1949).

Stahl (1938) has shown a dispersion of the red pigment of the decapod

Leander adspersus following the injection of extracts of the entire heads of the isopods *Mesidothea, Porcellio* and *Oniscus* and a concentration of the same pigment following the injection of extracts of the heads of *Idothea baltica.* Carstam and Suneson (1949) observed both a dispersing and a concentrating effect upon the red pigment of *Leander* following injection with extracts of the heads of *Idothea neglecta.*

Evidence indicating the presence of structures in *Oniscus asellus* which are apparently homologous to the sinus glands of other Crustacea (Walker, 1935; Stahl, 1938) has suggested the advisability of further investigations of the chromatophorotropins of isopod Crustacea. In addition, Gabe has shown in *Oniscus asellus* (1952a) and in *Sphaeroma serratum* (1952b) histological variations in the sinus glands during the intermolt cycle suggesting the physiological intervention of the gland in this cycle. However, its role in color change was not considered.

The wide variations in color observed in certain species of terrestrial isopods seemed to indicate the possibility that the progressive fixation of pigment, occurring as an adaptation to the terrestrial environment, may not be complete in these forms.

The purpose of this investigation was to determine the degree of chromatic activity occurring within these isopods and, if possible, to cast some light on the nature and distribution of the chromatophorotropins involved in these responses.

The authors would like to acknowledge their gratitude to John R. Cortelyou for his suggestion of the problem and his keen interest and aid throughout the course of this work.

Materials and Methods

Specimens of *Trachelipus rathkei,* a terrestrial isopod, were collected in large numbers from wooded areas in the vicinity of Chicago, Illinois. The animals when collected exhibited wide variations in color ranging from a mottled yellow-brown to an intense black. Males were normally much darker than females. Stock supplies of animals were maintained in covered battery jars containing moistened pieces of decaying wood. These conditions allowed for the successful maintenance of the stock in the laboratory for two to three months. Animals to be light-adapted were placed into clear Petri dishes lined with moistened filter paper. The dishes were then kept on a white background under constant overhead illumination. Animals to be dark-adapted were maintained in a similar manner in Petri dishes painted with black enamel. Attempts were made to establish a relatively constant humidity within the dishes, through the addition of water to the filter paper.

1. Animals

Microscopic examination of the pigmentary system of adult *Trachelipus* reveals that the coloration of the animal is dependent upon the relative distribution of two types of pigment within an apparently syncytial network of chromatophore processes. Although, in many instances, each pigment would appear to be contained within a distinct chromatophore network, the yellow usually lying beneath the black, the presence of extensive dichromatic areas indicates that the syncytium

is complete. Observations of the external surface of the intact dorsal segments showed a cuticle pattern which did not permit clear identification of the chromatophore structure. On the other hand, the internal surface is obscured by a considerable amount of adherent muscle. Histological preparations shed little light on this problem, since the yellow pigment is soluble in acid fixatives and the decalcifying agents used for these studies. At this time, no definite conclusions can be drawn as to the exact structural nature of the pigmentary system of the adult *Trachelipus*.

However, observations of larval *Trachelipus* prior to and immediately following their release from the brood pouch of the female show that in this species there are initially discrete chromatophores (Fig. 1) which rapidly develop into syncytial networks. These appear to be dichromatic and retain this condition during the early stages of fusion. Unfortunately, culture methods were not adequate to maintain the young animals long enough to observe the subsequent development of the chromatophore network.

The nervous system of *Trachelipus* is typically crustacean in nature and appears to be identical to that described for *Oniscus asellus* Linneas (Walker, 1935). The system is relatively unspecialized. The division of the crustacean brain into protocerebrum, deuterocerebrum and tritocerebrum is only weakly apparent in these forms. The protocerebral ganglia are clearly defined and are continuous with the well developed optic tracts. At the distal end of each optic tract in the region of the lamina ganglionaris, there is attached in a slightly ventrolateral position, a small oval structure which is bluish-white when viewed with reflected light. Upon injury this structure is seen to release a bluish viscous fluid, which diffuses through the saline. Both from its morphological appearance and its topographical relationship it resembles the sinus glands of other Crustacea (Fig. 2) and is referred to as such in the subsequent discussion. The deuterocerebrum is greatly reduced and appears as a narrow strip lying behind the protocerebral ganglia. The tritocerebrum, which bears the nerves leading to the antennae, is continuous with the short circumesophageal connective on each side. No tritocerebral commissure was observed although it may have been lost during the dissection. The subesophageal ganglion is not clearly defined and includes the first and second maxillary ganglia and the maxipedal ganglion. The nerve cord is characterized by a uniformity of segmentation of the thoracic ganglia and an anterior fusion of abdominal ganglia. The abdominal nerve cord thus consists merely of the posteriorly directed nerves arising from the fused ganglia.

2. Preparation of extracts and method of assay

For the preparation of extracts, the entire nervous system was removed from the animal with the aid of a dissecting microscope ($\times$ 45), and transferred to a syracuse watch glass containing Van Harreveld's solution. Animals from which the nervous systems were taken ranged in length from 9 to 11 mm. Care was taken to remove the entire system in one piece, such that the sinus glands were removed from the animal while retaining their connection to the optic tracts. The surrounding tissue was dissected away in order that extracts would include only the tissue of the sinus glands or of the neural elements.

To determine the relative distribution of the chromatophorotropically active

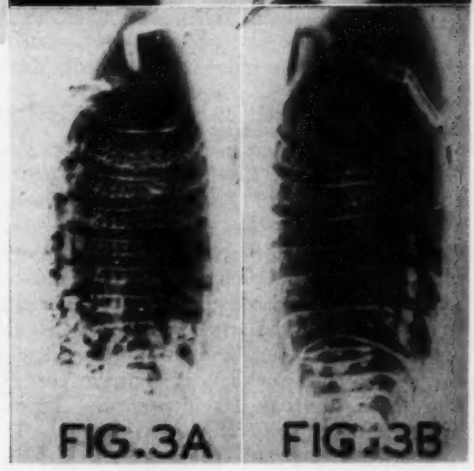

FIGURE 1. Discrete chromatophores of larval *Trachelipus*. (A) × 150; (B) × 440, illustrating early stages of concentration in response to light stimulus.

FIGURE 2. Dissected head of *Trachelipus* demonstrating sinus glands and cerebral region. (× 45)

FIGURE 3. Responses of *Trachelipus* to light. Adult female *Trachelipus* after being maintained in constant (diffuse overhead) illumination while on a light background (A), and after being maintained in total dark (B). (× 13.5)

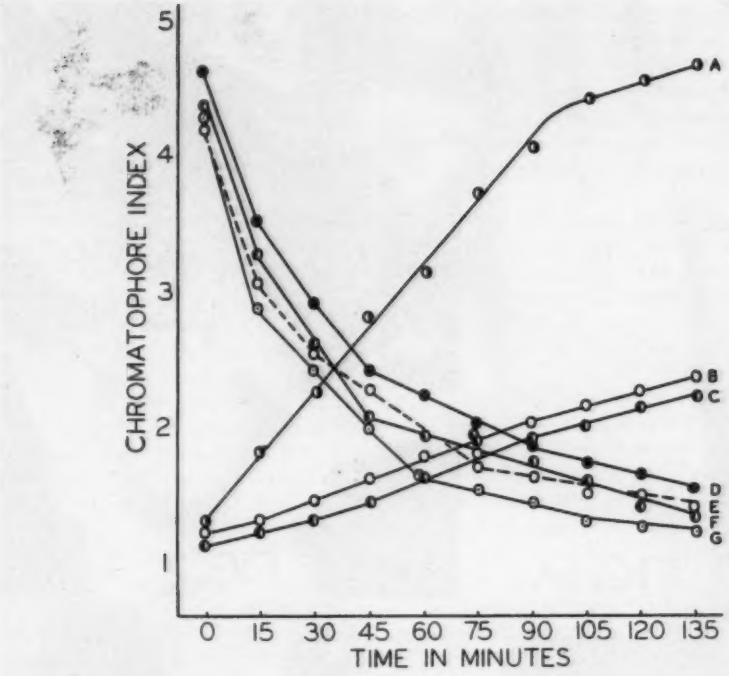

FIGURE 4. Comparative activities of extracts prepared from the sinus glands and neural elements of *Trachelipus* when assayed upon the red chromatophores of *Cambarus* (concentration 5). Dispersing activity: sinus glands (A); optic tracts (B); cerebral ganglia (C). Concentrating activity: subesophageal ganglia (D); anterior half of thoracic cord (E); connectives (F); posterior half of thoracic cord (G).

substances, extracts were prepared from nervous systems which had been divided into the following regions: (1) sinus glands; (2) optic tracts; (3) cerebral ganglia (including deuterocerebrum and protocerebrum); (4) circumesophageal connectives (including the tritocerebrum); (5) subesophageal ganglia (including first and second maxillary and the maxipedal ganglia); (6) anterior half of thoracic cord; (7) posterior half of thoracic cord (including the abdominal ganglion). Due to the extreme fragility of the abdominal nerves, no determinations were made of the activity of these elements. The individual fragments were placed into separate mortars. Because the chromatophorotropins undergo a slow inactivation at room temperature, the mortars were placed on ice during the preparation of the extracts. When 10 intact nervous systems had been dissected and divided into the appropriate regions, the fragments were triturated and mixed with one cc. of saline. The extracts were then maintained in small capped vials and refrigerated until further use. Extracts could be kept in this manner for several days without undergoing a loss of activity.

For use in experiments designed to determine the relative effects of concentration, the extracts were prepared in the same manner, from nervous systems divided into two portions, the first of which included the sinus glands, optic tracts and cerebral ganglia; the second included the connectives, subesophageal ganglion and entire thoracic cord. The number of nervous systems used in the preparation of these extracts varied according to the experiment and will be discussed with the appropriate results. In specified instances, extracts were prepared from entire nervous systems, including the sinus glands.

Since boiled extracts showed no significantly marked change in activity, the extracts in most cases were not boiled.

To alleviate the technical difficulties involved in observations of the pigmentary system of *Trachelipus*, assays were made upon isolated portions of the carapace of *Cambarus* sp. The carapace was removed from the animal and divided into pieces approximately 5 × 5 mm. in size. Since the effect upon the chromatophores seemed to proceed from the periphery to the center of the piece, more uniform results were insured by using pieces of approximately the same size. These were then placed into hanging drop slides containing a known concentration of extract. Into each depression were placed pieces of the carapace removed from: (1) a light-adapted animal; (2) an animal whose eyestalks had been removed at least 12 hours before the experiment was begun. In this way, the effectiveness of each extract was determined upon chromatophores which were initially fully dispersed as well as upon those which were completely concentrated. Controls consisting of carapace fragments maintained in Van Harreveld's solution were used in each experiment. The condition of the red chromatophores was then determined at 15-minute intervals using a standard chromatophore index (Slome and Hogben, 1928) where 1 signifies maximum concentration of pigment and 5 maximum dispersion with 2, 3, and 4 indicating the intermediate conditions. Since a difference was noted in the reaction of the chromatophores, average values were determined. Because this difference was more pronounced in older animals, small crayfish having a carapace length of 1.5 to 2.5 cm. were used. Each individual experiment was repeated four to five times.

Extracts of *Cambarus* sinus glands and central nervous systems were used to indicate that the chromatophores of isolated pieces of carapace retain their activity for several hours.

In another series of experiments extracts were injected into young intact *Cambarus*. One type of extract included sinus glands, optic tracts, and cerebral ganglia, while a second consisted of connectives, subesophageal ganglia and thoracic cords. The individuals receiving these extracts were (1) light-adapted for 24 hours and (2) eyestalkless, prepared 24 hours before the time of injection.

Experiments and Results

1. Responses to light

Adult *Trachelipus* exhibit slow and weak chromatic responses to light. Animals maintained upon a light background under the stimulus of diffuse light become slightly but perceptibly lighter than animals maintained in the dark (Fig. 3). More intense illumination, however, results in a darkening of the animals, even

upon a white background. In these cases the additional factor of an increase in temperature was also involved.

The discrete chromatophores of larval *Trachelipus* exhibit rapid and strong responses to light. Under the stimulus of light, the pigment of these cells rapidly concentrates. Although in many instances this results in the typically punctate condition of the chromatophores, in several cases the pigment apparently concentrated within the chromatophore processes, thus presenting a beaded condition. This reaction to light declines rapidly with age, in correlation with the progressive fusion of the chromatophore network.

2. Presence and distribution of chromatophorotropins

To determine the presence and relative distribution of the chromatophorotropic factors, extracts were prepared of sinus glands alone, as well as of isolated pieces of the central nervous system, as described in the preceding section. Since 0.5 cc.

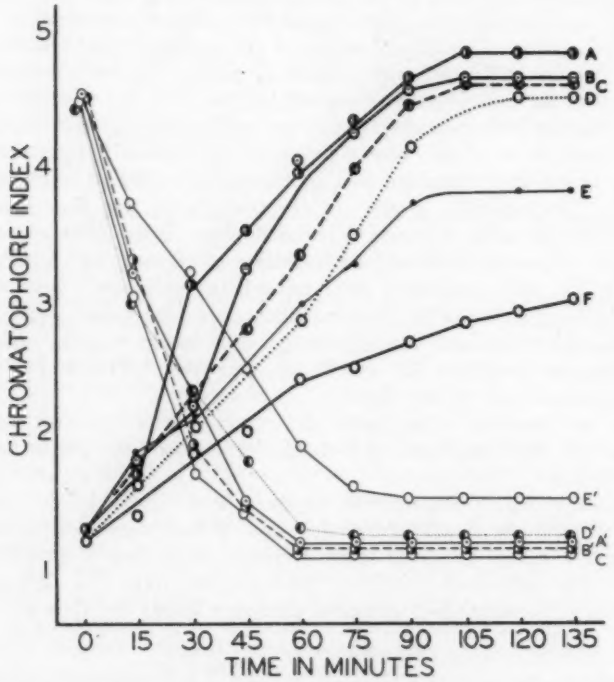

FIGURE 5. Effect of changes in concentration on the rate and magnitude of responses of the red chromatophores of *Cambarus* induced by extracts prepared from the sinus glands, optic tracts and cerebral ganglia of *Trachelipus*. (A—conc. 15; B—conc. 10; C—conc. 5; D—conc. 2.5; E—conc. 0.5; F—conc. 0.25.) Similar effects induced by extracts prepared from the connectives and thoracic cords of *Trachelipus*. (A'—conc. 15; B'—conc. 10; C'—conc. 5; D'—conc. 2.5; E'—conc. 0.5.)

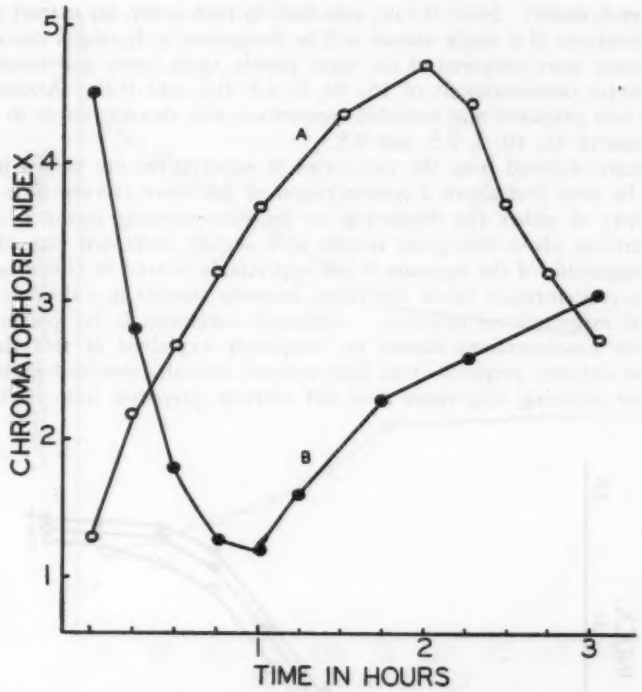

FIGURE 6. Average response of eight young *Cambarus* to injected extracts of (A) sinus glands, optic tracts, and cerebral ganglia in a conc. of 2.5; (B) connectives and thoracic cord in a conc. of 2.5.

of each extract was assayed upon isolated pieces of the carapace of *Cambarus*, the effective concentration included the specified structures from five animals.

An average of the results obtained from six identical experiments are presented in Figure 4. It can be seen that the sinus glands contain a substance which induces a dispersion of the red pigment of *Cambarus*. Whether the weak dispersing action of optic tracts and cerebral ganglia is due to the active production of this factor or merely to a diffusion through the tissue could not be determined by the method employed.

A similar number of experiments using extracts of the connectives or any segment of the thoracic cord demonstrated a concentration of the red pigment (Fig. 4). Since the individual fragments elicted approximately the same responses the substance controlling the concentrating action would seem to be relatively uniform in its distribution throughout the various regions of the cord.

3. Effect of concentration

In order to determine the relative effectiveness of the two chromatophorotropic factors, assays were made of various concentrations of the organs demonstrated

to contain each factor. Since 0.5 cc. was used in each assay, an extract prepared from the structures of a single animal will be designated as having a concentration of 1. Extracts were prepared of the sinus glands, optic tracts and cerebral ganglia in effective concentrations of 15, 10, 5, 2.5, 0.5, and 0.25. Another series of extracts was prepared and included connectives and thoracic cords in effective concentrations of 15, 10, 5, 2.5, and 0.5.

The results obtained from the two series of experiments are shown in Figure 5. It can be seen that above a concentration of 2.5 there is very little increase in the activity of either the dispersing or the concentrating factor. Increasing the concentration above this point results in a slightly increased rate of activity while the magnitude of the response is not appreciably altered in either case. Decreasing the concentration below this point, however, results in a decrease in both the rate and magnitude of response. Although variations in the potency of extracts at low concentrations cannot be completely explained at this time, it is possible that extracts prepared from light-adapted animals contained slightly more of the factor inducing dispersion than did extracts prepared from dark-adapted animals.

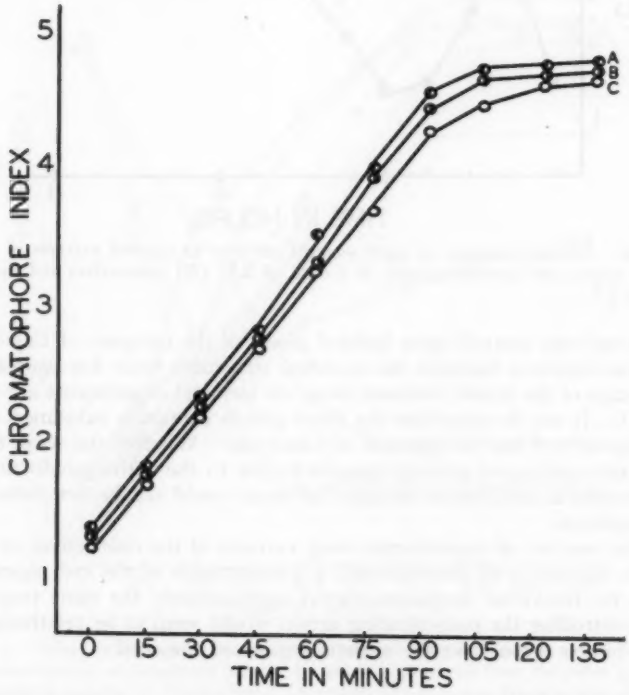

FIGURE 7. Comparative dispersing activity of extracts prepared from: A—sinus glands and entire nervous systems; B—sinus glands, optic tracts and cerebral ganglia; C—sinus glands alone. All extracts were prepared in a concentration of 5.

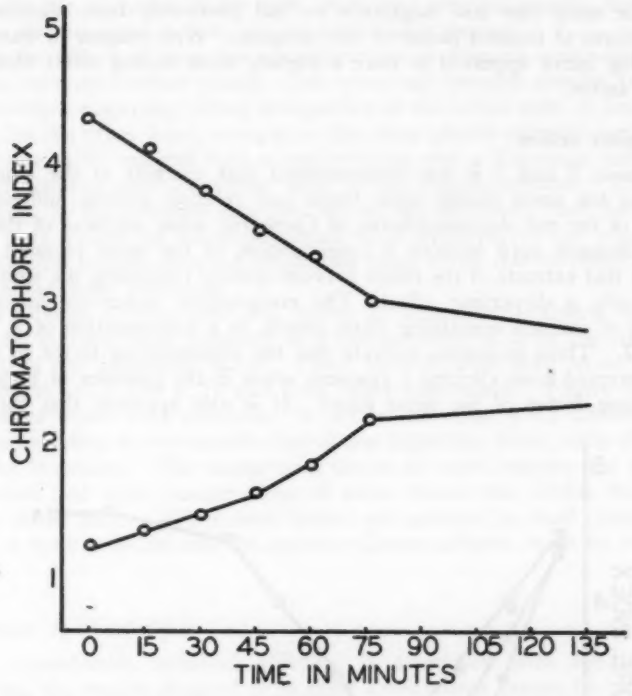

FIGURE 8. Dual response of the red chromatophores of *Cambarus* following perfusion with dilute (conc. 0.25) extracts of sinus glands plus the entire nervous system of *Trachelipus*.

A comparison of the two families of curves obtained (Fig. 5) indicates that the potency of the dispersing factor decreases more rapidly with decreasing concentrations that does that of the concentrating factor. A difference in the rate of activity of the two factors was also noted, since, within the range of concentrations studied, maximum dispersion was never attained in less than 90 minutes while complete concentration could be achieved in less than one hour. These two observations suggest that the factor eliciting concentration is stronger than that inducing dispersion. However, when extracts prepared from the entire nervous system including the sinus glands were assayed, only a dispersion of the red pigment was achieved. These conflicting observations suggest the existence of an antagonistic action between the two factors.

Since these studies, made upon isolated pieces of the branchiostegite, gave no indication of the duration of the responses obtained, eight young intact *Cambarus* were injected. Light-adapted *Cambarus* were injected with an extract of sinus glands, optic tracts and cerebral ganglia, while eyestalkless *Cambarus* were injected with extracts of connectives and thoracic cords. Both extracts were prepared in a concentration of 2.5. The responses obtained (Fig. 6) are of es-

sentially the same rate and magnitude as had previously been attained by the chromatophores of isolated pieces of the carapace. With respect to duration, the concentrating factor appeared to have a slightly more lasting effect than did the dispersing factor.

4. Antagonistic action

In sections 2 and 3 it was demonstrated that extracts of the sinus glands alone, or of the sinus glands, optic tracts and cerebral ganglia, induced only a dispersion of the red chromatophores of *Cambarus* while extracts of the connectives and thoracic cord induced a concentration of the same pigment. It was also shown that extracts of the entire nervous system (including the sinus glands) produced only a dispersing effect. The comparative dispersing action of the three types of extracts containing sinus glands, in a concentration of 5, is shown in Figure 7. These responses indicate that the concentrating factor in the nerve cord is prevented from eliciting a response when in the presence of high titers of the dispersing factor of the sinus gland. It is also apparent that the nervous

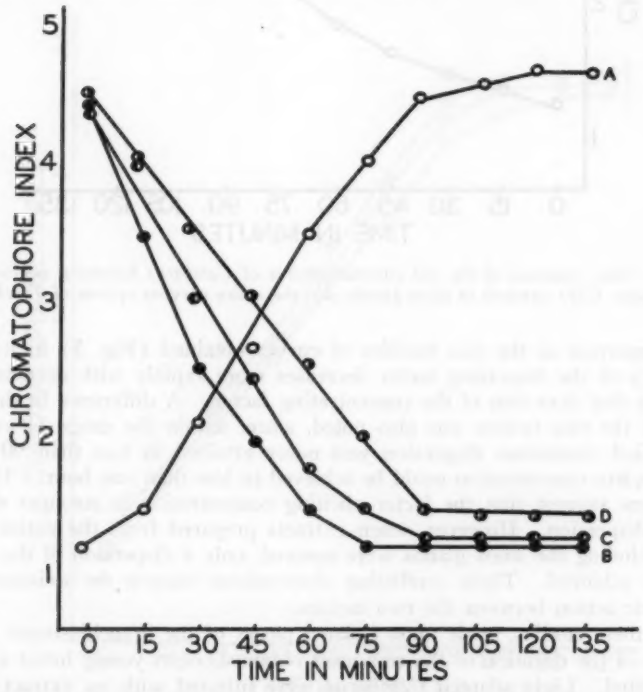

FIGURE 9. Further demonstration of the mutual antagonism existing between two chromatophorotropins of *Trachelipus*. A—dispersing factor alone (conc. 5). B—concentrating factor alone (conc. 5). C—concentrating and dispersing factor in a ratio of 4:1. D—concentrating and dispersing factor in a ratio of 3:2.

system, with the exception of the optic tracts and cerebral ganglia, does not contribute to the dispersing activity to any appreciable degree.

When the effects of very low concentrations of extracts were studied it was found that extracts of sinus glands, optic tracts and cerebral ganglia (conc. 0.25) produced only a dispersing effect, irrespective of the initial state of the chromatophores. On the other hand, extracts of the sinus glands plus the entire nervous system (conc. 0.25) exerted both a concentrating and a dispersing influence (see Fig. 8) dependent upon the initial state of the chromatophores. These results give support to the evidence that two factors are involved, since both types of response were elicited by the same extract when employed in low concentrations. The differential effects observed would not seem to be due to differing concentrations of the same substance.

In an attempt to determine the degree of antagonism existing between the two factors, extracts were prepared in a concentration of 5, of the concentrating factor and of the dispersing factor. These extracts were then mixed in various concentrations (4:1 and 3:2) and their effect determined. Figure 9 illustrates the responses which were obtained. It can be seen that when the concentration of the concentrating factor exceeds that of the dispersing factor, only the effect of the former is evident. The antagonism shown to exist between the two factors demonstrates that high concentrations of either factor will inhibit the effects of the more dilute factor. When both factors are present in small quantities, each produces a weak response and the chromatophores affected reach an intermediate condition.

5. Responses of Trachelipus

Since considerable technical difficulty is associated with the injection of *Trachelipus,* the results obtained from these experiments cannot be considered as conclusive. Due to the decreased viability of the animals and to the difficulty of observation following injection, the responses could not be readily interpreted. In general, the following effects were initiated.

Injection extract	Response of *Trachelipus*
Cambarus sinus glands	darkening
Trachelipus sinus glands	lightening
Trachelipus nerve cords	darkening

Although the results obtained must be considered as inconclusive, they suggest that the responses of the chromatophores of *Trachelipus* are directly opposite to those of the red chromatophores of *Cambarus.*

Discussion

The sinus glands of *Trachelipus* have been shown to contain a substance inducing a dispersion of the red pigment of the decapod *Cambarus,* and possibly a concentration of the melanin of the isopod *Trachelipus.* A dispersion of the red pigment of the decapod *Leander* following injection with extracts of the entire heads of terrestrial isopods *Mesidothea, Porcellio,* and *Oniscus* has previously been reported by Ståhl (1938). On the other hand, concentration of the melanin of

the isopod *Idothea* following injection of head extracts of the terrestrial isopod *Armadillidium* was observed by Okay (1945).

The results presented here indicate that the sinus glands at least of terrestrial isopods, although anatomically very similar to those of decapods, elaborate a substance inducing effects opposite to those elicited by extracts of the eyestalks of *Palaemonetes*-like decapods.

It has been shown by previous work (Koller and Meyer, 1930; Suneson, 1947; Carstam and Suneson, 1949) that eyestalk extracts of decapod Crustacea induce melanin dispersion in certain isopods. Evidence presented in this work, while only suggestive, indicates that the response in *Trachelipus* to *Cambarus* extracts is therefore like that of *Idothea*. In this respect, the isopods examined resemble the brachyuran Crustacea. This suggests an apparent difference in the reactive system as well as in the chromatophorotropins elaborated by the sinus glands of the two groups. However, there would appear to be a functional similarity, since extracts of the sinus glands of either group, when assayed upon their normal reactive system, are found to induce a concentration of the predominant pigment type.

The presence of a second and antagonistic hormone controlling the melanophores of isopods was suggested by Smith (1938) working with *Ligia oceanica*. He concluded that the background responses of *Ligia* are dependent upon the presence of two antagonistic hormones, one inducing a dispersion of the melanin, and the other a concentration of the same pigment.

Extracts of the connectives and thoracic cord of *Trachelipus* were found to induce a concentration of the red pigment of *Cambarus*, and possibly a dispersion of the melanin of *Trachelipus*. This factor, which induces an effect opposite to that produced by extracts of the sinus gland, is almost uniformly distributed throughout the connectives and ventral nerve cord.

A mutual antagonism has been shown to exist between the two chromatophorotropins of *Trachelipus*, at least in respect to their effect upon the red pigment of *Cambarus*. The direction and magnitude of the response is the result not only of the concentration of each factor, but, more directly, of the ratio between these concentrations. In this way a sufficient excess of either factor prevents the more dilute factor from eliciting a response. On the other hand, very dilute extracts known to contain small quantities of both factors were observed to induce responses in either direction dependent upon the initial condition of the chromatophores. The chromatophores perfused with these extracts attained a final condition of intermediate dispersion. Extracts prepared from the entire nervous system, including the sinus glands, have been shown to induce only a dispersing effect at high concentrations, while weak responses in both directions were elicited by dilute extracts. The rate of response is determined to a considerable degree by the concentration of the extract, but again is more closely correlated with the ratio between the two antagonistic factors present within a given extract. In regard to this point, it should be noted that the subesophageal ganglion and the connectives are located within the head of terrestrial isopods and, therefore, extracts prepared from the entire head would be expected to contain both factors. Due to the observed antagonism between the two factors, the weak activity of the optic tracts and cerebral ganglia does not necessarily preclude the possibility that both factors may be present within these tissues.

The effect which these observed properties of the two chromatophorotropins have upon the normal responses of the isopod must remain, for the present, hypothetical. It is interesting to note, however, the close correlation between the two chromatophorotropins of *Trachelipus*. Should this second factor be subsequently shown to exist within the nervous systems of isopods in general, the dispersion of the melanin observed to occur following the stimulation of the dorsal ocelli (Smith, 1938) may be due to the elaboration of high titers of this substance.

The identity or lack of identity of this factor with the *Crago*-darkening hormone found to be uniformly distributed throughout the central nervous system of *Idothea* (Brown and Saigh, 1946) also remains to be determined.

Although the observed chromatic responses of *Trachelipus* were slight, they appear to follow the same pattern as those observed in other species of isopods. However, the slight physiological color changes induced either by alterations in light stimulus or by the injected extracts could not be the sole explanation of the wide variations in color existing among these animals. Morphological color changes must necessarily assume an important role in the coloration of the adult animal. On the other hand, the ability of larval *Trachelipus* to exhibit strong physiological responses to light suggests that the loss of reactivity of the pigmentary system is a function of the progressive fusion of the chromatophore network with age.

In general, it can be stated that the progressive decrease in the reaction of the pigmentary system of *Trachelipus* is not accompanied by a corresponding loss of the chromatophorotropins of the sinus glands and central nervous system. It is possible that the hormones controlling physiological color changes may also be of functional significance in the control of the morphological color changes observed in these animals.

Summary

1. The terrestrial isopod *Trachelipus rathkei* was found capable of exhibiting weak physiological color changes in response to changes in background. Under the stimulus of diffuse light, animals maintained upon a light background became lighter than animals maintained in the dark. Under the stimulus of more intense light, the animals darkened even upon a light background.

2. Structures showing a marked resemblance to the sinus glands of decapods are located at the distal end of each optic tract and agree in position with the sinus glands of other isopods.

3. Extracts of the sinus glands of *Trachelipus* induce a strong dispersion of the red pigment of *Cambarus*.

4. Extracts of the optic tracts or cerebral ganglia induced a weak dispersion of the red pigment of *Cambarus*, while extracts of the connectives or any segment of the thoracic cord induced a strong concentration of the same pigment.

5. A comparison of the influence of change in concentration upon the effectiveness of the two chromatophorotropins indicates that above a minimal concentration, the rate of response is only slightly increased, whereas the magnitude of response remains unaltered. Below this level, both the rate and magnitude of response decline with decreasing concentrations.

6. An antagonistic action was found to exist between the two chromatophorotropins such that high concentrations of either factor prevented the more dilute

factor from exerting its influence. When very dilute extracts known to contain small quantities of both factors were assayed, both responses were elicited, such that the chromatophores assumed a condition of intermediate dispersion.

7. The response of *Trachelipus* to injected extracts proved inconclusive. The initiated responses suggest that the reactions of the pigmentary system of *Trachelipus* to injected extracts are opposite to those of *Cambarus*.

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VARIATIONS IN TEMPERATURE AND LIGHT RESPONSE WITHIN A PLANKTON POPULATION

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Zooplankton tends to concentrate about a particular depth, this depth being characteristic of certain external conditions, of the species concerned, and frequently also of the stage of maturity and brood of the organism. Diurnal and seasonal rhythms in external conditions are frequently reflected in vertical migrations of the animals, and geographical gradients in such factors as light and temperature have been shown to be associated with correlated changes in the vertical distribution of the species concerned. There is an extensive literature on the subject, that part pertaining to the Crustacea having been well reviewed by Cushing (1951). He describes also the experiments on tropisms, taxes, etc., which have been made in an attempt to understand the behavior observed in the field. Although widely differing and sometimes contradictory results have been obtained by different workers, it appears that such differences are characters of particular species rather than of whole groups. So far as it is possible to visualize a typical copepod, etc., it appears that the general pattern of behavior is much the same at least in copepods, chaetognaths, siphonophores and pteropods (Moore *et al.*, 1953). With the exception of larval fishes, which rarely exhibit diurnal migration, it is probable that the principles are generally applicable to such zooplankton as are capable of vertical movements.

Cushing shows how light and temperature are generally accepted as the major environmental factors controlling vertical distribution, with only a minor part played by salinity, CO_2 and other factors. There are many instances where, during most of the 24 hours, the plankton migrates vertically as if it were following a level of optimal illumination. On the other hand, there are instances of a thermocline proving a barrier to the vertical movement of a species, and the upper or lower limits of some species have been shown to follow closely the seasonal movements of particular isotherms (Nikitine, 1929). Vertical movements in response to light and temperature stimuli have been demonstrated experimentally, although the results sometimes appear to conflict with observations of natural behavior. It has also been shown that the nature of the response to light may be modified by the temperature.

Laboratory experiments, at least with marine zooplankton, suffer from the difficulty of reproducing natural conditions. Comparatively few marine zooplankton have been successfully kept for any long period in the laboratory. The ecologist has the uncomfortable feeling that the more successful laboratory species are the least typical, and can be reared only because of this. There are factors such

[1] Contribution No. 756 from Woods Hole Oceanographic Institution; Contribution No. 137 from the Marine Laboratory, University of Miami.

as food, previous treatment, internal rhythms and others which may well modify behavior. On the other hand, any attempt to analyze behavior under natural conditions suffers from the large number of simultaneously varying factors, none of which can be controlled. It has the advantage, though, that the animals may be assumed to be behaving typically, and that, particularly in the tropics, a large number of species may be studied, so that the pecularities of atypical species will not seriously affect the observations. The present paper is a study of the relative responses to temperature and light of some zooplankton in the Florida Current, and how these vary in a vertical section through the plankton population.

Material and Methods

The material was collected in a region of the Florida Current lying from ten to forty miles east of Miami, Florida. This is ocean water with isotherms sloping very steeply across the current so that a wide range of temperatures is available at any depth. Seasonal variations in the current pattern and velocity result in a wide range in conditions at any one station throughout the year. An account of the hydrographic conditions at the ten mile station has already been published by Miller *et al.* (1953) and the methods used in collecting plankton were described in the same paper. The material was drawn from samples, collected for other purposes, in part under a joint program of the Marine Laboratory of the University of Miami and the National Geographic Society, and in part by the Marine Laboratory under contract number NObsr-57146 for the Office of Naval Research. The analyses were carried out under the latter contract and under Bureau of Ships contract number NObsr-43270 at Woods Hole Oceanographic Institution. The siphonophore examination was carried out in part by the author and in part by D. C. Roane (1954). The chaetognaths were examined by H. Owre and her results will be included in a doctorate thesis at the University of Michigan. To all of these the author wishes to express his indebtedness.

The present work is restricted to the period within about two hours either side of noon, when the illumination is not changing rapidly, and it is assumed that the plankton has had time to stabilize at the depth at which it is found. Vertical temperature sections were available for each station. The vertical distribution of illumination was calculated by methods previously used (Moore, 1950) and from these the depths of selected isotherms and isolumes were obtained. For each station, the percentage vertical distribution of each species was calculated as follows. First, the count from each of the oblique net hauls was scaled to the equivalent of a tow of one mile, the factor being obtained from the trace on the depth-distance recorder towed along with the nets. Each count was assumed to be applicable to a column of water reaching from half way between the mid-depth of that tow and the mid-depth of the tow above it to half way to mid-depth of the tow below it. The top tow was assumed to terminate at the surface, and the lowest at the deepest point shown on its trace. Integration of the whole column was performed by multiplying each count by the length of water column to which it applied, and adding the products. By the use of a calculating machine, the levels were found corresponding to 10%, 20%, etc., of this total. These, referred to as the 10% level, etc., are the depths above which this percentage of the species occurred on the particular occasion. Values from 10% to 90% were

calculated, but the 0% and 100% levels were not sufficiently clearly defined to be usable.

The following species, used in previous work, were present in sufficient numbers to be significant. It should be noted that, with the inclusion of more stations, some of the regressions obtained differ from ones previously quoted.

SIPHONOPHORA

Chelophyes appendiculata (Esch.)
Diphyes bojani (Esch.)
D. dispar Chamisso & Eysenhardt
Eudoxoides mitra (Huxley)
E. spiralis (Bigelow)
Abylopsis eschscholtzii Huxley
A. tetragona Otto.
Bassia bassensis (Quoy & Gaimard)

CHAETOGNATHA

Sagitta enflata Grassi
S. hexaptera D'Orb.
S. lyra Krohn
S. bipunctata Quoy & Gaimard
S. serratodentata Krohn
S. minima Grassi
S. decipiens Fowler
Pterosagitta draco (Krohn)
Krohnitta subtilis (Grassi)

The various species characteristically occupy different levels. In order that the data for the various species could be combined, the mean 10%, 20%, etc., levels were calculated for each species, these being the means of all stations. For each station, the results were then expressed as deviations from the mean on that occasion, a positive value being deeper, and a negative shallower. The deviation of a typical siphonophore on that occasion was the mean deviation for the eight species available, and that of a typical chaetognath was the mean for nine species. For comparison, the levels of the 15° C. isotherm and of the 10^{-2} isolume were similarly expressed as deviation from their respective means.

After testing for correlations between the deviations of the plankton and the isolume and isotherm, and finding that these were significant, partial regressions were calculated with the results shown in Figures 1 and 2. The regression coefficients show the extent of vertical movement of the plankton in relation to a one-meter shift in the 15° C. isotherm or 10^{-2} isolume. In all cases the coefficients are positive, that is to say, the plankton movement is in the same direction as the movements of the isotherm and isolume.

For simplicity it may be assumed that the temperature and light graphs in the above figures are straight lines. If the regressions of plankton movements on isotherm and isolume movements are referred to as temperature and light responses, then the regressions of the responses on percentage level can be calculated. From these, the levels occupied by the typical siphonophore or chaetognath may be predicted for any given hydrographic conditions for this region. As a verification of the goodness of fit between such predicted levels and those actually observed, six stations were selected which offered a particularly wide range of hydrographic conditions. Figures 3 and 4 show the comparison of the values from the 10, 50 and 90% levels as predicted and as observed. These cover a more than five-fold range of depth under different conditions, and the agreement appears to be good.

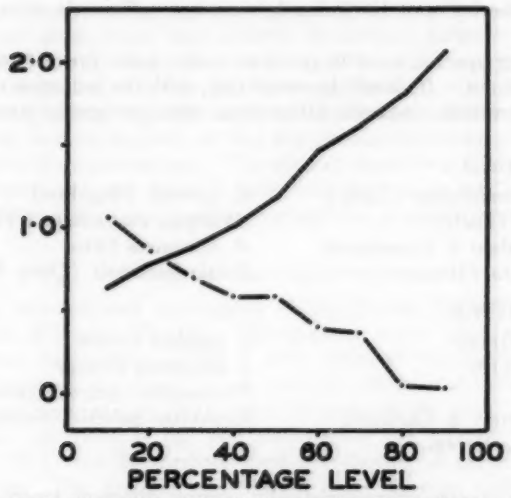

FIGURE 1. Typical siphonophores. Partial regression of plankton movement on movement of the 15° C. isotherm (whole line) and of the 10^{-4} isolume (broken line), and the variation of these regressions with percentage level; 0% represents the top, and 100% the bottom of the plankton column.

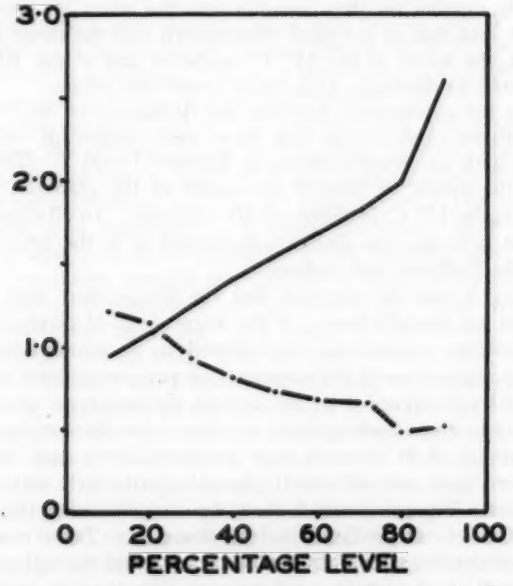

FIGURE 2. Typical chaetognath regressions indicated as in Figure 1.

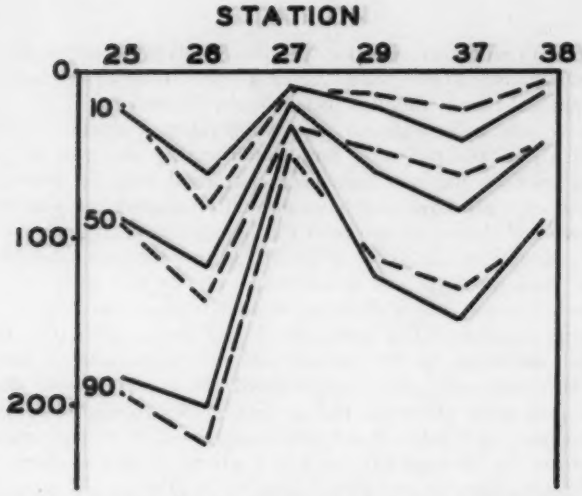

FIGURE 3. Typical siphonophore. Comparison of the observed depths, in meters, of the 10%, 50% and 90% levels at six stations (whole lines) with the depths predicted by the regressions referred to in the text (broken lines).

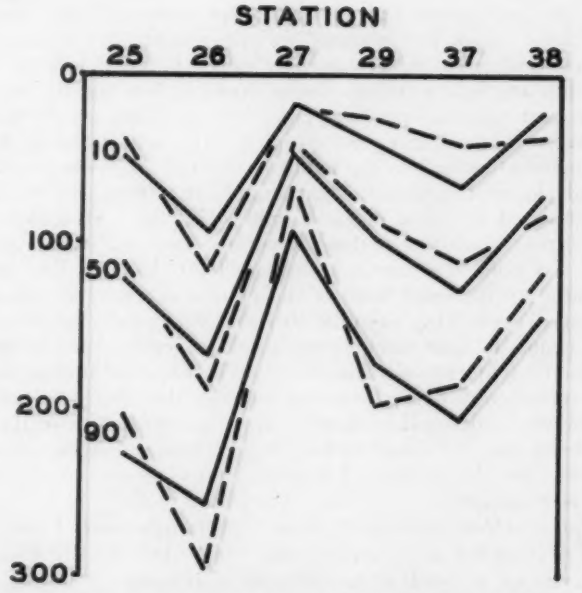

FIGURE 4. Typical chaetognath comparisons indicated as in Figure 3.

Discussion

Cushing (1951) summarizes many examples of plankton whose day level varies on different occasions. Where these variations have been correlated with changes in an environmental factor, this has usually been illumination. The Florida Current provides an unusually wide range of temperature variation at depth, and so may be particularly advantageous for demonstrating the role of temperature. Further, most previous workers have been concerned with the level of maximum concentration of the plankton and have not differentiated between the behaviors of the shallower and deeper members of the population of a given species. In an earlier study (Moore, 1953; Moore *et al.*, 1953) in which considerably fewer data were available than in the present work it was shown that there was an increase in temperature response and a decrease in light response in the lower levels of the plankton as a whole. The same trend was indicated in the four separate groups studied, but many of the values could not be considered statistically significant. J. B. Lewis (in press) has studied the euphausids of this area, and shown that illumination plays the major part in controlling both day level and vertical diffuseness, with little or no demonstrable effect of temperature. Roane (1954) compared the average day level of a group of siphonophores at two stations in the Florida Current and found these to be 79 and 253 meters. The corresponding extinction coefficients were 0.062 and 0.051 while the depths of the 15° C. isotherms were 151 and 619 meters, respectively. It seemed probable that the great range in depth of the animals was associated with both illumination and temperature differences. The present work shows that the observed vertical distributions can be accounted for if both temperature and light play a part in regulation of level, and if it is assumed that their effects are additive.

Two characteristics of the graphs in Figures 1 and 2 call for explanation. The temperature and light responses change more or less linearly with percentage level, and the regressions of plankton movement on isotherm and isolume movement may have values well in excess of unity. The author has, so far, been able to find little information on how the nature or strength of a reaction varies among the individuals in an invertebrate population. The frequency distribution may perhaps be expected to follow either a symmetrical or a skewed normal curve. The latter is true in what is perhaps a parallel case, the variations in rate of hemolysis of red cells in a sample (Ponder, 1930, 1932). Too much reliance cannot be placed on the exact form of the present curves, and unfortunately the ends of the curves are lacking, even the 10% and 90% values probably being based on too small counts to have much significance. It seems clear, though, that they cannot represent a frequency distribution rising to a marked peak at any particular value. Subsequent work on single species suggests that they may, with more data available, prove to be flat-peaked curves. Another explanation of their shape lies in the probability that, as shown below, temperature and light are not sufficient alone to account for the observed behaviors, and a further environmental factor will have to be considered.

The extent to which the regression coefficients may exceed unity can hardly be attributed to error due to inadequate data. They indicate that the animals may respond to a change in depth of an isotherm or isolume by moving considerably further. In other words, although the movements of the animal are in such direc-

tion as could maintain them at a constant temperature or illumination, they do not, in fact congregate at the depth corresponding to such a constant value. Finally, very much poorer correlations are obtained if an attempt is made to relate the depth at which the animals are found to *in situ* temperatures and illuminations, than if the correlations are made between the depth changes of the animals, the isotherms and the isolumes. All this suggests that a third factor, related to depth, and which we tentatively suggest may be pressure, must be included in the complex. Although pressure has received much less consideration in this connection that the other two factors, there is some evidence that it may affect vertical movement of zooplankton (Hardy and Bainbridge, 1951).

The similarity of Figures 1 and 2 gives support to the statement already made that there may be a pattern of depth control mechanism typical of widely different animals. Within the species which we have grouped together, specific differences may be expected both in the strengths of the responses and in the relative response to light and temperature. Such would be in agreement with the very different behavior patterns observed in the many forms which have been worked with. We have found a marked difference in response in different parts of a population. There is no evidence as to whether this represents permanent differences among the individuals or whether it represents a sorting, resulting from varying responses, of individuals whose responses fluctuate continually. Differences between individuals may be expected, but so may changes in the reactions of a single individual. The latter might be more or less random, they might vary in relation to previous condition or to some control such as a feeding cycle, or they might vary with an inherent diurnal or other rhythm. Whatever may prove to be the case, a significant point seems to emerge in connection with the interpretation of experiments with plankton. Firstly, if the experimental animals are not always collected from the same part of the plankton column then differences may be expected in the nature of the experimental behavior. Secondly, experiments made with animals taken from the top or bottom of the population may yield results which are not applicable to the main concentration of the population.

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MITOCHONDRIAL STRUCTURE IN PARAMECIUM AS REVEALED BY ELECTRON MICROSCOPY

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The structure of the mitochondria as revealed by electron microscopy has received considerable attention recently, and from these studies there have emerged several general descriptions of the ultrastructure of these cell particulates. Common to all the descriptions is the belief that mitochondria possess a membrane which is distinct from the internal matrix and which constitutes the external edge of the structure. The structures found in the interior of mitochondria have been variably interpreted as having ridges arising from the membrane projecting into a central matrix (Palade, 1953), parallel plates or membranes extending from side to side (Sjöstrand and Rhodin, 1953), concentrically arranged lamellae (Beams and Tahmisian, 1954), or parallel rods (Glimstedt and Lagerstedt, 1953). Our study of mitochondrial structure in *Paramecium*, based upon sections manipulated in a manner slightly different from those in most of the above studies, leads us to conclusions concerning structure which are different from any presented up to this time. A preliminary report of these has appeared (Powers, Ehret and Roth, 1954).

Materials and Methods

1. *Organisms*

Clonal cultures of two species of *Paramecium* were employed. The principal stocks examined were 51 VIII kk (Sonneborn) of *P. aurelia* and So 6 C (Chen) of *P. bursaria*. The organisms generally were used near the end of a growth cycle, very few food vacuoles being present in the cells at the time of fixation.

2. *Fixation*

The organisms, after having been concentrated by slow filtration through sintered glass, were fixed in 1% OsO_4, buffered at pH 7.4 with 0.045 *M* citrate-phosphate buffer (McIlvaine's), for 30 to 60 minutes at about 26° C. At the end of the fixation period the animals were carried through a series of fluids (without centrifugation) as follows: distilled water, 2 changes, 5 minutes each; 50% ethyl alcohol, 15 minutes; 75% ethyl alcohol, 30 minutes; one part absolute alcohol, one part methacrylate mixture, 30 minutes; methacrylate mixture, 3 changes, one hour each. The methacrylate mixture was 40% ethyl methacrylate and 60% n-butyl methacrylate with the hydroquinone inhibitor removed. After the third change, the plastic was irradiated with ultra-violet light for 12–16 hours at 40–45° C.; this treatment produced complete polymerization without the use of chemical catalysts.

3. Sectioning and mounting

Sections were cut with a glass knife in an International Minot thin-sectioning microtome equipped with a worm gear mechanism allowing a minimum thickness of 1/40 μ. Sections in the range 1/10 to 1/40 μ were used for observation. The solution used to float the sections was either 20% dioxane or 20% ethyl alochol. The sections were allowed to float on the solution for 10–20 minutes at 30–40° C. before being mounted on formvar-covered grids. Some of the sections were studied with no further preparations. Others were soaked briefly in toluene to remove the plastic. This treatment consisted of immersing the grid with the membrane-supported section for 20–30 minutes at room temperature.

4. Microscopy

An RCA type EMU-2A electron microscope was used. The microscope was equipped with the standard objective pole piece using a 60 μ objective aperture or with the wide-angle pole piece using rear focal plane objective aperture. The micrographs were taken at magnifications of 900–9200 diameters, further magnification being accomplished photographically.

Results

Identity of the particles

The particles studied here conform, in chemical characteristics, to descriptions of mitochondria given by other authors: they are osmiophilic and under certain physiological conditions they stain blue-green after Janus Green B, and pink after 2,3,5 triphenyl tetrazolium chloride. In addition they have been shown by Thomson (personal communication) to be associated with several oxidative enzymatic activities ascribed to mitochondria in other forms.

Size and shape

In crushed unfixed preparations under phase optics, the mitochondria appear as small spheroids about 0.7 μ in diameter. In 10% sucrose solution they also appear as spheroids. However, sections of mitochondria (Figs. 4–17) after osmic fixation appear to have been derived from cylinders with rounded ends, as well as from spheroids.

We believe that the spheroid bodies seen under phase optics in freshly crushed preparations may present the geometric shape of many of the particles in the living condition, because, as well as can be seen, the particles beneath the pellicle of the intact cell just before crushing appear spheroidal. If the spheroidal shape predominates in the living material, it is apparent that processing has caused a change in the shape of many of the particles from spheroids into cylinders and hyperboloids-of-one-sheet, with rounded ends, and with lengths about twice their widths. The volumes of the two structures are about the same (a cylinder 0.5 $\mu \times 1.0$ μ, compared to a sphere 0.70–0.75 μ in diameter).

It is certain that fixation with OsO_4 results in some changes in size of organelles within these cells. The trichocysts, evident in Figures 2, 3, 4, and others, are larger than they are in the living cell. This fact is demonstrated by a comparison of Figure 1 with Figure 2. Figure 1 shows a group of trichocysts just

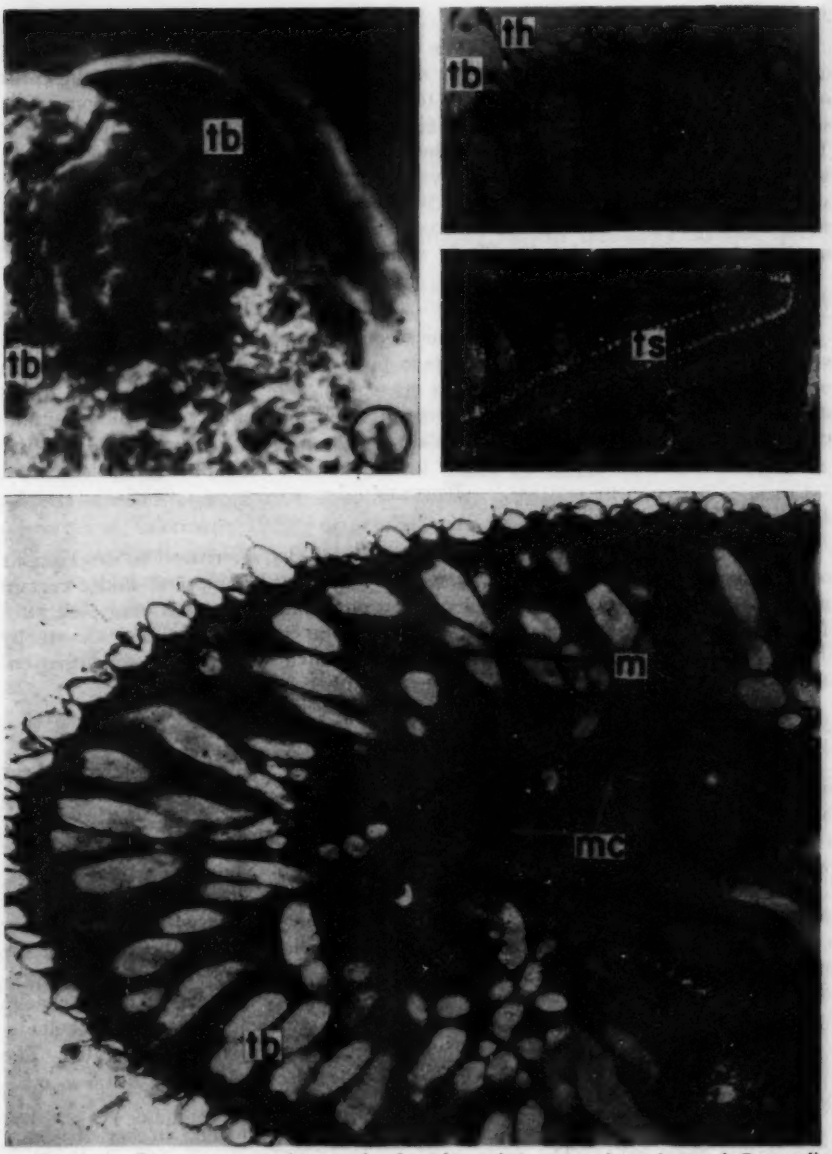


FIGURE 1. Phase contrast photograph of surface of compressed specimen of *P. aurelia* showing size of body of trichocyst relative to head in living condition. 1000 ×.

m, mitochondrion; mc, macronucleus; mcm, macronuclear membrane; mt, mitochondrial tubule; n, nucleolus; p, pore of mitochondrial tubule; pl, pellicle; pt, plastic; s, peritubular space; tb, trichocyst body; tc, trichocyst cap; th, trichocyst head; ts, trichocyst shaft ("exploded").

beneath the pellicle in a freshly crushed preparation. The tip length: body length ratio in this preparation, and in all others like it, is about 2: 3. In the electron micrograph of sections of osmium-fixed material, the tip appears to retain its live dimensions, but the body, in all the preparations we have examined, is enlarged with a resulting ratio of 2: 5 or more. In a few preparations complete shaft formation is seen even within the cell boundaries. So, as far as these structures are concerned, the fixation procedure is evidently not the best. In regard to others, however, the fixation procedure appears to be very good. The close adherence of the cytoplasm to the macronuclear membranes (Figs. 4, 5, 9) is in contrast to the situation observed after fixation of *Paramecium* by an agent such as warm Schaudinn's solution, which causes the appearance of an artificial vacuolar space separating the macronucleus from the cytoplasm. In regard to the mitochondria, it is probable that, in addition to the change in shape, some swelling of the structures occurred also, *i.e.*, in the undisturbed state the bodies may be slightly more compact.

Distribution

Figure 5 demonstrates that mitochondria in *P. aurelia* are, with certain exceptions, most abundant near the pellicle of the animal. The relationship between distribution of mitochondria and the reproductive stage of the cell will be discussed in a subsequent report.

Structure

Figures 6–17 present the evidence for our idea of mitochondrial structure in *Paramecium*. Figure 6 is typical of the appearance of sections which are not soaked in toluene before examination. It may appear from this photograph that the mitochondrion is a sac bounded by a continuous membrane as postulated by others for metazoan and protozoan mitochondria. The central area (the "mitochondrial matrix" of other authors) appears clear and is approximately equivalent in density to the structureless ground substance representing the cytoplasm in these sections. Against this background, osmiophilic structures (the "microvilli" of Sedar and Porter, 1954) are found. Also in this section it is to be noted that the pellicle appears to be a double structure, and that the cytoplasm is homogeneous except for widely scattered dense granules.

Figure 7, from a section in which the plastic remains, demonstrates the same mitochondrial structure. It is presented to show in addition the appearance of the tip of the trichocyst and the surrounding cap when viewed in section with the plastic-in. This is to be compared with Figure 8, an adjacent section which was soaked in toluene for 30 minutes before examination. The tip of the trichocyst and the surrounding cap are sharply defined, in contrast to the fuzzy, ill-defined representation in Figure 7. Furthermore, no detail is visible in the space between the tip and the cap when the plastic is in, although threadlike structures extending between the tip and the cap are clearly visible after toluene treatment of the sec-

FIGURE 2. EMG (electron micrograph) of section of *P. aurelia*, demonstrating enlargement of trichocyst body relative to head after fixation with OsO_4. 5000 × Plastic removed.

FIGURE 3. EMG of fully extended trichocyst in cytoplasm. 10,000 ×. Plastic removed.

FIGURE 4. EMG of section of *P. aurelia*. Autogamous animal showing distribution of mitochondrial relative to the trichocysts and the macronuclear fragments. 4000 ×. Plastic removed.

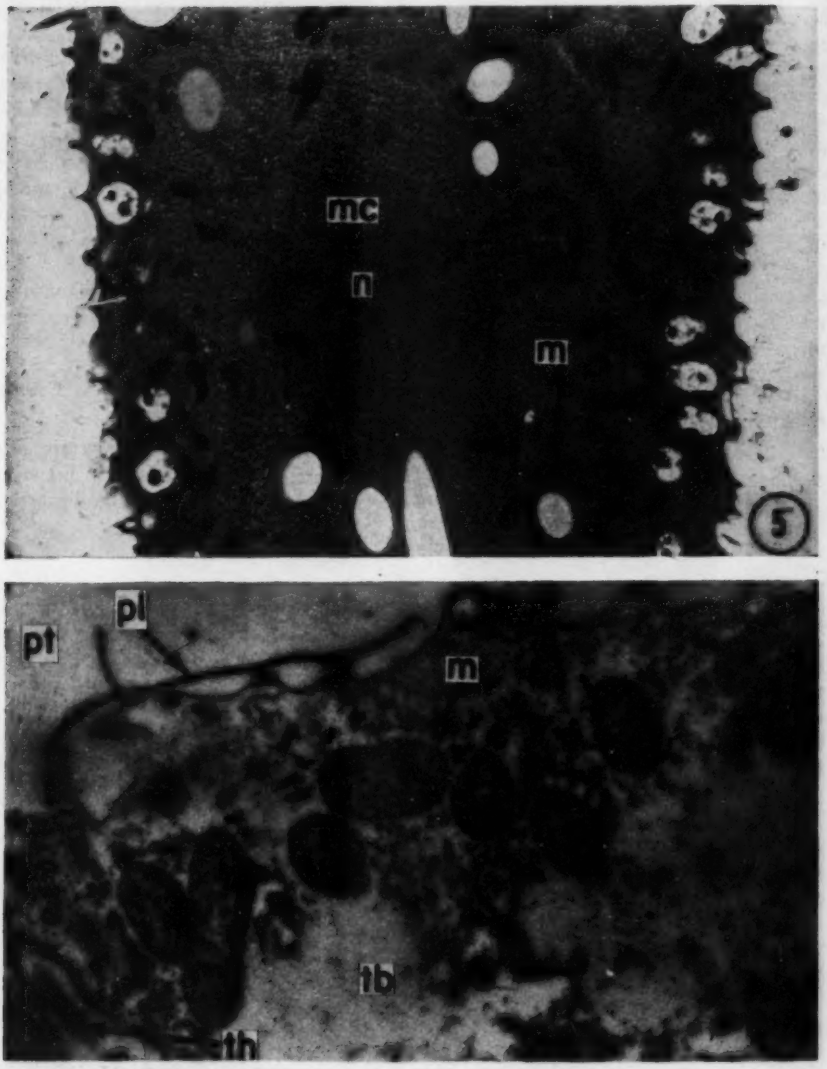


FIGURE 5. EMG of section of *P. aurelia* showing peripheral distribution of mitochondria in cytoplasm. Macronucleus containing nucleoli visible in center of section. 5000 ×. Plastic removed.

FIGURES 6 AND 7. EMGs of sections of *P. aurelia* with plastic remaining in. The cap, head, and body of the trichocyst are to be compared with corresponding structures in Figure 8. Note that the body of the trichocyst is hardly distinguishable from the plastic outside the pellicle. 20,000 × and 26,000 ×.

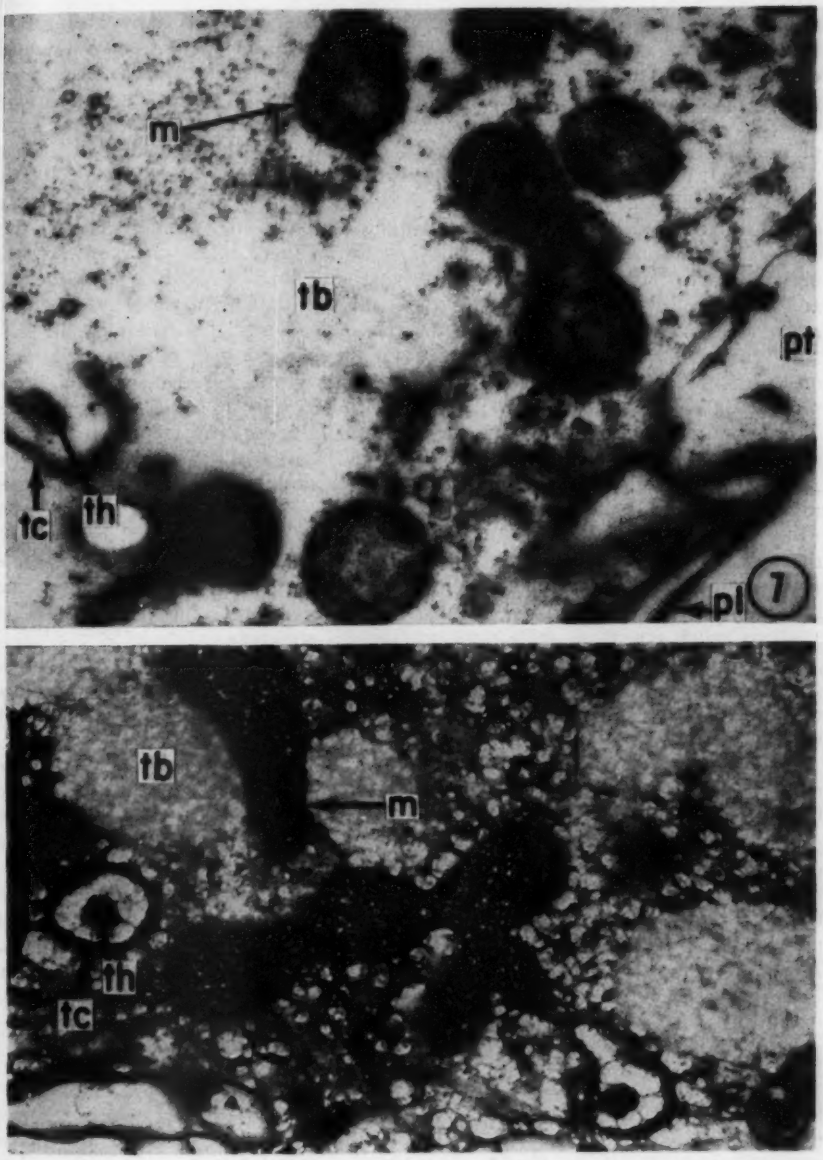


FIGURE 8. Section similar to those in Figures 6 and 7, except that toluene treatment preceded examination. This section came from the same block as those in the two previous figures. 26,000 ×. Plastic removed.

tion. The body of the trichocyst in Figure 7 is homogeneous and structureless; in Figure 8 the body is seen to have a grainy appearance. The ground substance of the cytoplasm in Figure 8, in contrast to Figure 7, is net-like, vacuolated, and contains particles of varying density (or osmium content). The frequency of occurrence of the densest particles indicates that they are identical with the cytoplasmic particles seen in Figure 7, but the net-like, less dense structures cannot be seen in Figure 7 because of masking by the plastic.

The mitochondria in Figure 8 are seen to consist of an osmiophilic material that is interrupted by numerous circular and elliptical interspaces. There is no external membrane distinct from this material. There is no clear central area but, rather, material that is less dense than some portions of the "outside edge" and material surrounding many of the interspaces. The more dense material about the spaces is continuous with the less dense material surrounding it.

Figures 9–17 are additional examples of sections through mitochondria demonstrating their ultrastructures. All the sections present essentially the same picture. There is an osmiophilic substance constituting the mass of the particle. This substance appears to be continuous throughout all sections except for very small interspaces. The interspaces appear as sharply defined circles, ellipses, or straight or contorted canals. Arrangements of the interspaces are not altogether random, for patterns are evident in the form of apparent concatenation of circles (Fig. 11), and whorled (Fig. 9) and anastomosing (Figs. 9, 10, 17) canals. All the sections show that the mitochondrion consists of this osmiophilic substance permeated by numerous small canals, irregularly but definitely arranged. The mitochondria of *P. bursaria* (Figs. 12, 13) appear more compact than those of *P. aurelia*, but otherwise show the same general structure.

The size of these interspaces was measured from greatly enlarged pictures. In a series of 30 circles, which were considered cross-sections of the interspaces, the mean diameter was 15 mμ with a standard deviation of 0.9 mμ. The range was 7.9 mμ to 25.2 mμ.

At first glance some of the sections (Figs. 16, 17) appear to show that the mitochondrion is surrounded by a membrane distinct from the interior, resembling the membrane of the macronucleus (Fig. 9). Closer examinations reveal, however, that the thick edge is clearly continuous with the material within the mitochondrion.

In many of the sections it can be seen that the interspaces are continuous with the cytoplasm, *i.e.*, an opening exists in the edge of the mitochondrion at that point. These holes are infrequent; they cannot be observed in most sections. However, numerous examples of them can be found and some are offered in Figures 14–17. Even when the plastic remains in the section, the structures visible in the matrix are occasionally seen to open to the outside (Fig. 7).

Discussion

The additional treatment of soaking the sections in toluene, which removes most of the plastic, reveals structure of the mitochondrion invisible in sections not so treated. On the basis of Roth's (1954) study of the effects of methacrylate on appearance of structure in sections, it is clear that the effect of the presence of the plastic is to mask certain detail in all parts of the cell, including the mito-

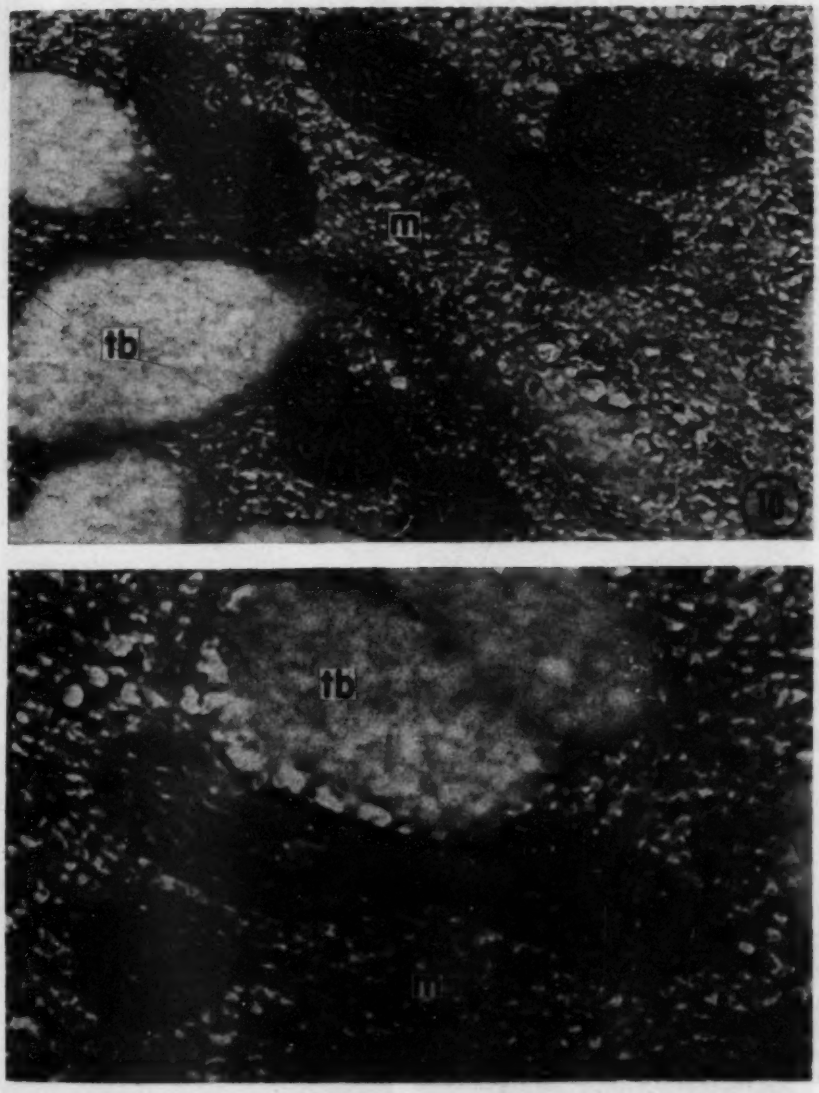
FIGURE 9. EMG of section of *P. aurelia*. On the right side is the macronucleus with its intact membrane. The details of mitochondrial structure are typical for *P. aurelia*. 42,000 ×. Plastic removed.

chondrion. All material of low electron scattering power ("density") is obscured by the plastic because it does not allow distinction between this low density material and those areas which appear as spaces in the sections prepared in the other manner. At the same time, the difference in effective density between the high density material and the low density material is greater, so that these areas are in sufficient contrast against the "structureless" background to be visible in the photograph (*e.g.*, some of the material immediately bordering the canals and the very dense granules of the cytoplasm). The result is that we see thin-walled "tubules" in the mitochondria and discrete, well-separated granules in the cytoplasm. The knowledge, then, that areas of low density are not distinguishable from those of no density and that areas of great density are the only ones seen when the plastic remains in the sections reconciles differences in appearance by the two techniques.

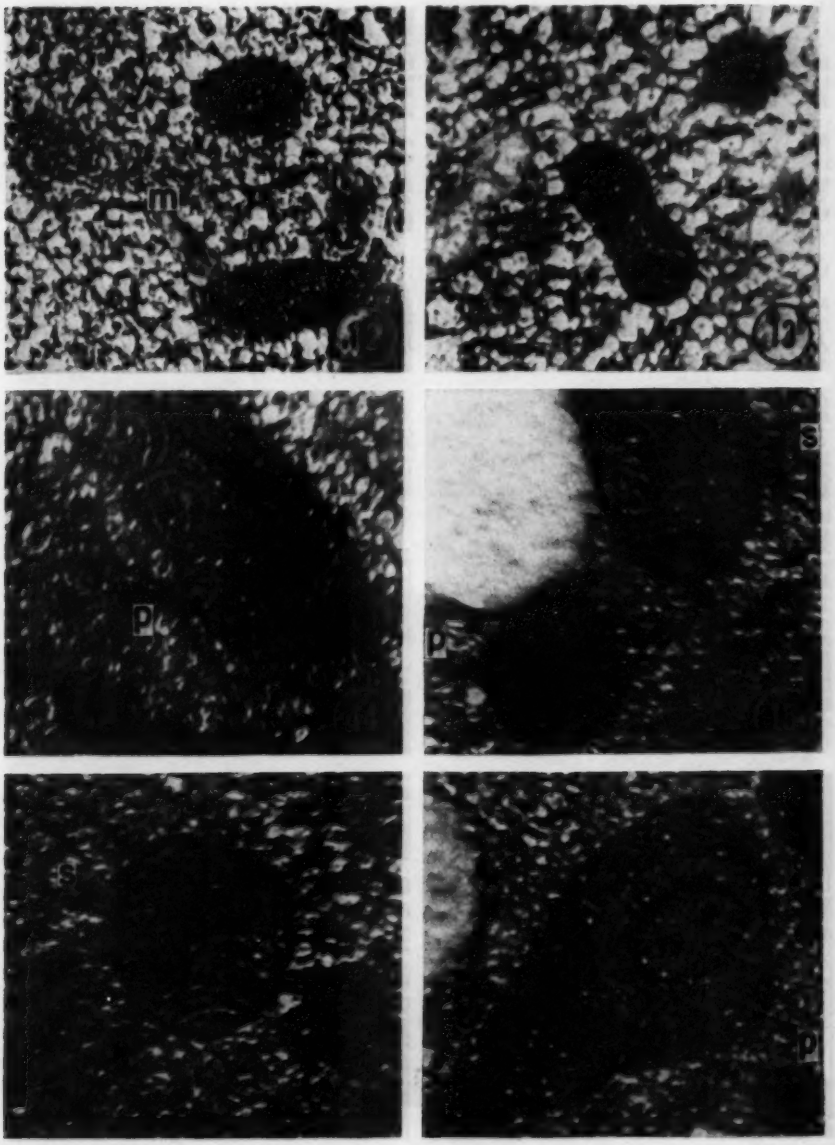
While removal of plastic may cause some displacement of ultrastructure (Hillier and Gettner, 1950), we propose that the structure of the mitochondrion is revealed only under these conditions. Removal of plastic from these sections results in cross-sections of cilia which seem collapsed to a slight extent because the double structure of the individual fibers reported by others (Fawcett and Porter, 1954) is recognized only with difficulty. But it should be noted that the change brought about by the removal of the plastic is a slight movement of structure, and this kind of change hardly accounts for the architecture observed in the sections of mitochondria after plastic removal. It is difficult to conceive that structure of such high degree of organization (comparable in all the details visible in sections before plastic removal) is the result of changes brought about by solvent action.

There are two other reports of structure of mitochondria of protozoa as revealed by electron microscopy, one in *Euglena* (Wolken and Palade, 1953) and the other in *Paramecium multimicronucleatum* (Sedar and Porter, 1954). These authors, using the generally accepted method of leaving the plastic in during examination, conclude that the mitochondria possess external membranes. In *Euglena* numerous ridges (called *cristae*) project from the "membranes" into an internal structureless matrix. The authors point out that this mitochondrion has a differentiated membrane similar to that observed in mitochondria of other animal cells. In *P. multimicronucleatum*, it is said, there are villi projecting from the external "membrane" into the internal matrix. The latter report is reconcilable with our observations in all respects, for, prior to being soaked in toluene, the sections show the same structure reported by these authors. In regard to the observation in *Euglena* (Wolken and Palade, 1953), we suspect that removal of the plastic might reveal that the matrix has real structure, and that this structure may have the same relationship to the "membrane" as that observed in our studies.

In other studies (Palade, 1953; Sjöstrand and Rhodin, 1953) mitochondrial membranes are described from sections of mammalian tissues in which the plastic remains during examination. Very clearly defined dense lines are visible about the particles, but close examination of many of the photographs shows that the membranes are frequently discontinuous and vaguely defined, and sometimes bear unexpected (for membranes) relationships to the structures found internally. In view of this and in view of our demonstration that plastic removal reveals



FIGURES 10 AND 11. EMGs of mitochondria of *P. aurelia* at high magnification showing typical structure. 69,000 × and 84,000 ×. Plastic removed.



FIGURES 12 AND 13. EMGs of sections of *P. bursaria* showing the same general mitochondrial structure as observed in *P. aurelia*. 26,000 ×. Plastic removed.

FIGURES 14–17. EMGs of sections of *P. aurelia*. Pores of mitochondrial tubules and peritubular space are shown. Figure 17 is an enlarged mitochondrion from Figure 9. 67,000 ×, 67,000 ×, 74,000 ×, and 67,000 ×. Plastic removed.

many details of structural interrelations not visible in other sections, the generalization that mitochondria have differentiated membranes is open to question.

Two general kinds of interpretation of the structure of the mitochondrion can be made on the basis of the observations reported here. The particle can be considered a continuum which is interrupted by numerous canals, the canals bearing the same relation to the substance of the particle as does the space made by a pin in a lump of clay to the clay itself. Or, it can be considered to be made up of a tightly packed collection of tubules (like hollow macaroni), the walls of which in some places fuse to form a continuum of osmiophilic substance. In other areas the tubular characteristic is retained.

Examination of the figures presented, especially in the regions of looser structure, leads us to favor the tubular interpretation. In this interpretation, the basic structure is a tubule with a lumen approximately 15 mμ in diameter. The innermost portion of the tubule wall appears very dense after osmium fixation; the outermost portion of the wall, except at the edge of the particle, is frequently less dense. These portions often are continuous with corresponding parts of adjacent tubules setting up a continuum not separable into component tubules. In places the tubules are evidently not continuous, forming what might be called the peritubular (or intertubular) space. At the edge of the particle this continuum is often dense. Infrequently, the tubules are seen to open through the dense edge of the particle, the lumen being continuous with the surrounding cytoplasm. The lumen itself is represented by a space in the photographs. Of course, this does not mean that this lumen is empty in the living condition; it does mean that any substance occurring there must be different from that of the wall of the tubule which reduces OsO_4 and becomes relatively dense.

This interpretation accounts for all details visible in both types of sections. With plastic in, the frequently dense edge and the dense linings of the tubules apparently constitute the entire structure. When the plastic is removed, the external "membrane" is seen to be a dense peripheral portion of tubule wall and the tubules are seen to consist of both dense and less dense material. A sac-like membrane,[1] such as seen in Figures 4, 5, 9, surrounding the macronucleus, cannot be demonstrated, although the presence of the macronuclear membrane itself indicates that toluene treatment did not remove any structure like it.

The relation of this tubular interpretation of mitochondrial structure in *Paramecium* to the descriptions given for certain other organisms is not direct, although certain similarities are recognizable. Glimstedt and Lagerstedt (1953) conclude from studies of isolated particles that in rat liver the mitochondrion consists of a collection of granules arranged in cords; and although there is no evidence that the cords are hollow (tubular), the method of preparation would not permit observation of a cavity even if it does exist, and these structures may, in fact, be quite similar to those described for *Paramecium*. In the case of the mitochondrion of the germ cell of *Helix* (Beams and Tahmisian, 1954), which consists of a group of concentrically arranged lamellae, the relationship is even less clear and direct. However, the development of a lamellar structure from tubular precursors is suggested by Leyon (1954) for the development of the grana of the choroplast in *Aspidistra* from the primary grana. A process like this (phylogenetically or

[1] We are distinguishing between boundary interfaces and the kind of structure demonstrated by cell and nuclear membranes.

ontogenetically) could unite conceptually such seemingly diverse mitochondrial microanatomy as that seen in *Paramecium*, *Helix*, and the rat. On the other hand, it is recognized that real morphological differences in mitochondrial structure may exist; for instance, the mitochondrion of the proximal convoluted tubule as interpreted by Sjöstrand and Rhodin (1953) might suggest another basic structure.

The observations of Weinstein (1954) of the structure of particulates called sarcosomes in sections of chicken heart muscle are directly comparable to those reported here. The sections are quite similar to those of *Paramecium* mitochondria from which the plastic was not removed. Longitudinal and cross-sections of tubules within these particles in the muscle tissue are easily observed in the photographs. Although Weinstein (as well as Leyon) interprets his photographs as showing sac-like membranes, they can as easily be interpreted as not demonstrating the presence of a membrane on the external surface. This particle of the chicken heart, then, becomes nearly identical in structure to the mitochondrion of *Paramecium*.

Based on chemical and physical observations made on the mitochondria of several organisms, certain inferences concerning structure appear in the literature. One opinion is that the behavior of mitochondria in hypertonic and hypotonic solutions, together with some information on permeability of sarcosomes, indicate the presence of a differentially permeable membrane about the mitochondrion. This opinion was most recently presented by Lindberg and Ernster (1954) who feel that the recent morphological evidence as presented by Palade (1953) and Sjöstrand and Rhodin (1953) is complete confirmation of the membrane theory, and is so final as to make any contrary opinion "hardly maintainable today." Another view (Harman, 1950; Green, 1952) is that the biochemical and biophysical evidence is not of such a nature as to require that mitochondria possess membranes. It had been proposed that a complex protein can exhibit the osmotic behavior observed with no unique requirement for fluid interiors surrounded by differentiated membranes. The evidence presented in this paper supports this suggestion, in that the mitochondrion is shown to consist of an almost continuous material which is not separated from the exterior by a membrane different from it. One other characteristic, not predictable from the biochemical evidence, is revealed—the continuous material is the substance of the walls of tubules, the lumens of which are sometimes seen to open to the outside.

The way in which the mitochondrion functions physiologically may only be guessed from the morphological evidence. The most simple thought is that reaction of enzyme and substrate occurs at the external surface of the mitochondrion. However, the existence of the tubules and their openings to the cytoplasm suggests that these reactions may occur within the lumens of the tubules. In this instance, the surface available for reaction is very much greater than in the former case; and if in addition the peritubular spaces are involved, the surface reaction area is even greater yet.

Summary

1. An electron microscopical study of the mitochondria in *Paramecium aurelia* and *P. bursaria* has been made from thin sections of cells imbedded in methacrylate

plastic. Some of these sections were studied directly (plastic-in), and others were soaked in toluene before examination (plastic-out).

2. Plastic removal reveals structural detail not visible when the plastic remains in the section. At the same time, the relationship between the newly observed structures and those seen in the other sections is accounted for.

3. The undifferentiated mitochondrion in *Paramecium* is interpreted to consist of a compact mass of twisted tubules, the walls of which are made up of at least two kinds of substances (based on density differences in the photographs). The lumen of the mitochondrial tubule is about 15 mμ in diameter and is seen at times to be continuous with the cytoplasm. A distinct feature of this mitochondrion is the lack of a demonstrable membrane distinct from the material of the walls of the tubules in contrast to some interpretations of mitochondrial structure in many other organisms.

4. The basic tubular structure of the mitochondrion in *Paramecium* is recognizable in the cytoplasmic inclusions (mitochondria, primary grana) of several phylogenetically distant organisms.

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THE FATES OF SEGMENTS FROM TUBULARIA PRIMORDIA[1]

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During the reconstitution of decapitated *Tubularia* stems, a conspicuous pigment precedes morphological differentiation in the primordial region. Accordingly, it was very early postulated, by Loeb (1892) and by Driesch (1899), that the pigment itself, as a "formative substance," may initiate or control differentiation of the hydranth. Recently, Goldman (1953) showed that at least a part of the pigment is synthesized within the primordium during reconstitution. Meanwhile, Davidson and Berrill's (1948) experiments with mutilated primordia showed that the pigment distribution can be used as a landmark by which primordia can be sectioned to give stems and fragments of abnormal but predictable behavior. That work suggested the possibility of clarifying the role of pigment in determination, by removing various pigmented and non-pigmented regions of primordia and allowing them to differentiate in isolation.

Material and Methods

For each experiment, a single colony of *Tubularia crocea,* from the same area off Woods Hole, Massachusetts, was used. Stems were selected for uniformity of diameter and appearance, and decapitated one to three millimeters below the hydranth. The proximal (aboral) ends were cut to give stems 7 to 10 mm. in length, and the stems were left in fresh sea water at 18° to 22.5° C. until a reasonable number had developed to the desired stage. Those stems were isolated and divided evenly into lots representing control and experimental groups. The desired segments were then removed with scissors under a binocular microscope, and isolated. Control groups consisted of unoperated stems. The water in which all stems and segments were kept was changed at least once a day. All data recorded represent the form of the hydranth at the time of emergence from the perisarc. Frequently, the regenerates were unable to emerge because of the absence of a point of attachment to the perisarc; in those cases, the regenerate was removed with dissecting needles for observation.

All data are recorded in tabular form, each table or figure headed by explanatory diagrams. The first vertical column at the left of each figure contains a diagram representing the stage worked with. Pigment distribution, where pigment is present, is indicated by stippling. The second vertical column consists of a series of diagrams showing the places from which segments were cut, such segments being indicated by diagonal lines. The horizontal row at the top of each figure contains diagrams representing the form of the regenerate at the time of

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emergence. A description of each of the types of diagrams appears in Figure 1. In each square of the following figures the number of individuals appears in standard lettering, while the percentage of the total is italicized.

Results

1. The development of random segments of varying length.

Pieces of primordia are, of course, considerably smaller than stem pieces customarily used in differentiation studies, and so it was necessary first to determine what effect small size itself might have on reconstitution. Accordingly, a number of segments of varying lengths were cut at random from normal stems, sorted into three size groups, and allowed to develop independently. Segments of about 0.25 mm. were never observed to differentiate, but always either died or rounded permanently into undifferentiated cellular clumps. Segments 0.5 to 0.75 mm. in length did better: of 60 cases studied, 8 (13.3%) formed complete hydranths, 3 (5%) formed bipolar hydranths, 2 (3.3%) lacked, or possessed reduced, proximal tentacles, and 47 (78%) either died or failed to differentiate. Of 34 segments approximately one millimeter in length, 8 (23.6%) formed complete hydranths, 3 (8.8%) formed bipolars, and 23 (67.7%) died or failed to differentiate. The forms described are diagrammed in Figure 1. Driesch (1897), following the development of short stem pieces apparently one to three millimeters in length, reported that 6.1% were unipolar, 31.7% bipolar, and 62.2% complete hydranths. In the experiment reported here, the percentages are 8.3, 25.0, and 66.7, respectively, if dead and undifferentiated segments are excluded from the calculations.

It is therefore evident that segments of small size as a rule form either complete hydranths, none at all, or bipolar hydranths. Hydranths in which a set of tentacles is missing ("unipolar hydranths") without being replaced with a duplicate of the other set are rare. Hence, small size tends largely to inhibit reconstitution completely, or to effect the production of bipolar hydranths.

2. The development of segments from the prepigmentation stage (stage II)

Figure 2 shows the development of segments removed from stems two hours after decapitation, and before any pigmentation could be observed. The terminal

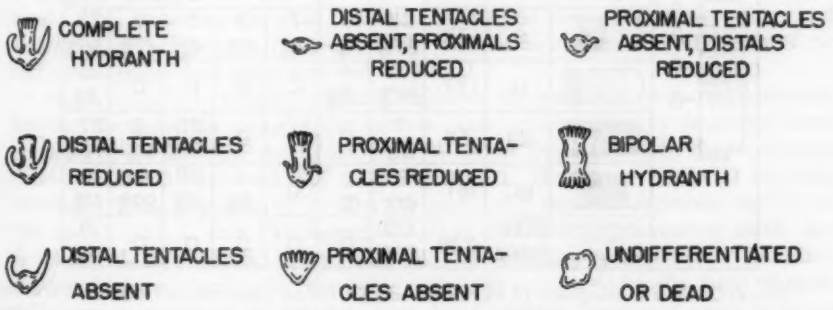

Figure 1. An explanation of the symbols used in the figures.

STAGE	SITE OF SEGMENT	SEGMENT	NUMBER				
A B		A	74	0	1 1%	24 32%	49 67%
		B	73	73 100%	0	0	0
		CONTROL	72	68 94%	0	0	4 6%

FIGURE 2. The development of segments from the pre-pigmentation stage.

segments, 0.5 to 1.0 mm. in length, showed virtually no regulatory ability with respect to proximal tentacle formation, two proximal tentacles being formed in the case of a single segment. Regulation with respect to distal tentacle formation was complete in both distal and stem pieces.

3. The development of segments from the stage of diffuse pigmentation (stage III)

Figure 3 illustrates the development of segments from stems in which the pigment had begun to collect in the first three millimeters of the stems, but in which there was no sharp boundary between pigmented and unpigmented regions, one grading into the other. In stage III, as in stage II, no morphological differentiation is apparent. Segments representing the terminal 0.5 mm., which was not pigmented, failed to develop in 90% of the cases, evidently because of their small size. The development of distal tentacles in 18 individuals (10% of the

STAGE	SITE OF SEGMENT	SEGMENT	NUMBER							
A B C D		A	182	0	0	0	1† .5%	17† 9%	0	164 90%
		B	20	0	0	0	0	9 45%	7 35%	4 20%
		C	77	23* 29%	5 7%	7 9%	3 4%	3 4%	8 10%	28 36%
		D	177	171 97%	1 .5%	0	0	0	0	5 3%
		AB	56	1 2%	0	0	0	26 46%	2 4%	27 48%
		BC	161	66 41%	2 1%	0	1 .5%	28 17%	46 29%	18 11%
		CONTROL	228	219 96%	0	0	0	0	0	9 4%

FIGURE 3. The development of segments from the diffuse pigmentation stage. † Occurred in one experiment only and could not be repeated. * A 72-hour time lag in emergence was observed.

total) occurred in one experiment only, as noted by the dagger in Figure 3, and could not be repeated. The development of segments from other levels indicated that the ability to form distal tentacles resides in more proximal parts at this stage, and it is probable that in the erratic experiments cited the proximal cuts were made farther from the tip than intended, thus including some of the region of distal tentacle potency. Segments representing approximately the distal half of the pigmented region (B segments) formed distal, but not proximal, tentacles, while segments representing the proximal half of the pigment (C) formed an array of types that included distal and proximal tentacles in all combinations, as did segments comprising the entire pigmented region (BC segments). Note that a 72-hour time lag in emergence of the C segments occurred, and that 29% had produced complete hydranths by that time. The delay appears to represent a reversal of development, stimulated by cutting, and resulting in reorganization to form complete hydranths in those cases.

Segments comprising the distal half of the pigmented region plus the stem tip (AB segments) developed in much the same way as the B segments alone, indicating that the unpigmented stem tip is not important in reconstitution. The behavior of the BC segments, in forming no proximal tentacles 17% of the time, and of the C segments in forming unipolar distals in 3 cases, is taken to mean that the pigmented region of stage III ends at a region of transition between areas of differing abilities for proximal tentacle formation.

Stems from which all of the pigmented area was removed (D) formed complete hydranths in 97% of the cases.

4. The development of segments from the stage of defined pigmentation (stage IV)

Figure 4 illustrates the development of segments removed from primordia in which the pigment had become sharply defined at its proximal limit. As in the preceding stages, no morphological differentiation had occurred by that time. In addition to the large pigment band, which ended about 2.5 mm. from the tip of the stem, many stems also possessed a more narrow, darkly pigmented band which occupied the distal 0.25 mm. of the larger band. In order to determine any effect of the band on the development of the segments, some cuts were made so as to include it with the terminal, or A, segments: those are designated A+. B segments represent the distal half of the pigmented area, including the narrow band. B-C segments represent the entire pigmented area minus the narrow band, and the B-CD segments represent stems from which only the unpigmented tip and the narrow band were removed.

As in stage III, unpigmented tip segments showed very poor reconstitution, but tip segments that included the narrow band (A + segments) developed distal tentacles in 54% of the cases, and in 79% of the cases in which they contained the entire distal pigmented half (AB segments). However, the B-CD segments developed distal tentacles in 95% of the cases, and the B-C segments in 61% of the cases. The C and CD segments, which also lacked the narrow band, also developed distal tentacles, in 62% and 82% of the cases, respectively. Therefore, the distal, narrow band, which probably marks the area in which the distal tentacles will form, evidently is not necessary for distal tentacle formation: more proximal

stem levels can also form distal tentacles if called upon to do so, and with no loss of time. It will be seen later that this is a general ability that applies to all stages.

The development of the A, A+, AB, B, B-C and C segments, which represent various combinations of the pigmented and tip regions, indicated that, surprisingly, proximal tentacles were unable to form in any part of the pigmented region. Of 166 individuals represented by those segments, only two showed proximal tentacle formation. On the other hand, all pieces that included the stem proper, that is, pieces 7 to 10 mm. in length (B-CD, CD and D segments), of course developed proximal tentacles if they differentiated at all.

5. The development of segments from the tentacle band stage (stage V)

Figure 5 represents the development of segments removed from primordia that had developed to the stage in which the pigment was sharply distributed between two bands occupying the terminal 3.0 mm. of stem. Unpigmented tip segments (A segments) showed their typical inability to form tentacles. As shown by the figures for the B and BC+ segments, all of the segments that contained the distal pigment band formed distal tentacles if they developed at all. Of the segments that contained only the proximal band plus the proximal half of the unpigmented interband region (C-D), only 8 of 33 developed, and of those only one lacked distal tentacles. However, stems to which the proximal band and interband regions were left attached (CDE) developed in 10 of 14 cases, and of those, half lacked distal ten-

STAGE	SITE OF SEGMENT	SEGMENT	NUMBER							
A B C D		A	29	0	0	0	0	1 3%	0	28 97%
		A+	33	1 3%	0	0	14 42%	3 9%	0	15 46%
		B	29	0	0	0	22 76%	0	0	7 24%
		C	29	0	0	0	17 59%	0	1 3%	11 38%
		D	29	27 93%	0	0	0	0	0	2 7%
		AB	33	1 3%	0	0	22 67%	0	3 9%	7 21%
		B-C	13	0	0	0	6 46%	0	2 15%	5 39%
		CD	17	12 70%	2 12%	3 18%	0	0	0	0
		B-CD	20	14 70%	5 25%	0	0	0	0	1 5%
		CONTROL	43	43 100%	0	0	0	0	0	0

FIGURE 4. The development of segments from the defined pigmentation stage.

STAGE	SITE OF SEGMENT	SEGMENT	NUMBER						
A–E		A	53	0	0	0	0	1 2%	52 98%
		B	11	0	0	0	11 100%	0	0
		BC+	34	7 20%	0	0	24 71%	0	3 9%
		C-D	33	5 15%	0	1 3%	2 6%	0	25 76%
		CDE	14	5 36%	3 21%	2 14%	0	0	4 29%
		E	38	28 73%	4 11%	0	0	0	6 16%
		CONTROL	65	64 98%	0	0	0	0	1 2%

FIGURE 5. The development of segments from the tentacle band stage.

tacles or possessed rudimentary ones. It was repeatedly observed during the experiments that any segment, from any stage, can eventually form distal tentacles if it can differentiate at all. If the distal tentacles are absent or rudimentary at the time of emergence, it indicates that the process of distal tentacle formation has been arrested or delayed in starting.

The limitation of proximal tentacle potency, as the above data show, was more clear by stage V. In the case of the C-D segments, which consisted of the proximal band plus the proximal half of the interband region, of the 8 that developed, 6 possessed proximal tentacles. In the BC+ segments, however, proximal tentacles formed in 20% of the cases, even though they possessed no part of the proximal band, but only the distal band and half of the interband region. It therefore appears that the region of proximal tentacle potency extends a short distance distal to the limit of the stage V proximal band.

6. The development of segments from the proximal striation stage (stage VI)

When segments are isolated from advanced primordia in which the proximal band has developed tentacle ridges, proximal tentacles can develop only in segments which contain a part of the proximal anlage. A tendency in the same direction also holds true for the distal tentacles, since the absence of the distal band leads to the absence of distal tentacles from the regenerate. Usually, however, within another day, the incomplete regenerate belatedly begins to form a tiny set of distal tentacles. In the case of the proximal tentacles, however, once the hydranth has differentiated without them, they can never subsequently be re-formed. Thus, even *Tubularia* is not totipotent in all situations. This ability of mutilated primordia to reconstitute missing distal, but not proximal, tentacles was first observed several years ago, by Davidson and Berrill (1948).

Since primordia of stage VI showed the same range of regenerative abilities that the adult hydranth shows, data from the experiments are not included here.

Discussion

Ordinary stem segments seldom give rise to unipolar hydranths, whereas segments removed from primordia in various stages of reconstitution often do. The data cited above indicate that the nature of the affected structures and the degree of the effect depend upon the positions in the primordia of the regions incorporated into the segments, and upon the time at which the cuts were made. A regional change in regenerative potency occurs within two hours after removal of the hydranth from the stem, since a short segment that would ordinarily form a complete hydranth or a bipolar instead forms a unipolar hydranth possessing only distal tentacles, and no proximals. Thus, two hours after decapitation, the distal millimeter of stem can no longer support proximal tentacle formation. The single exception to this behavior, indicated in Figure 2, can be explained most reasonably by assuming that in that case the cut was made at a point of transition between areas of differing abilities for the support of proximal tentacle formation.

As Figure 3 shows, by the time the stage of diffuse pigmentation develops, further changes in the regenerative potential of the first three mm. of the stem have occurred: proximal tentacles can no longer form from the distal half of the pigmented region, while the proximal pigment halves can produce both distal and proximal tentacles in any combination of number or quality, depending upon the particular segment. It is reasonable to assume that the cuts, in those cases, fall at levels at which rather abrupt changes have developed along the stem with respect to the ability to form tentacles. On the other hand, cuts through the transition zones of later stages often lead to the production of fewer or rudimentary tentacles. Thus, although the transition from a determined to a non-determined zone is rather abrupt, there nevertheless appear to be short zones of gradual change along the axis, and the inclusion of small amounts of such gradient regions in a segment can thereby lead to quantitative manifestations in the hydranth. However, hydranths possessing such rudimentary tentacles ordinarily go on to form tentacles normal in both size and number in a few days. It is as if the adult hydranth can later furnish some missing quantity if a little is present in the operated segment.

The C segments of Figure 3 are interesting in that they indicate that the cutting operation may sometimes result in delayed emergence, accompanied by reorganization to form complete hydranths. Note that similar segments from stage IV (Fig. 4) were not delayed in emergence, and did not produce complete hydranths.

Since the distal pigmented halves of stage III (Fig. 3) do not form proximal tentacles, while the proximal halves may or may not, it appears that the critical cuts, which determine whether a particular segment will be able to form proximal tentacles, are those that separate the pigmented from the unpigmented parts of the stem. Since those cuts were made as precisely as possible to coincide with the limit of pigmentation, and since they produced a spectrum of tentacle types, it appears that the pigment limit of stage III marks a zone of transition with respect to proximal tentacle determination: proximal tentacles form only when the

cut happens to include enough of the presumably unpigmented proximal tentacle area. That area, therefore, appears to be immediately behind the pigmented region of stage III. Moreover, stage III stems from which all of the pigmented region has been removed go on to produce normal, complete hydranths, which emerge at the same time that the controls do. Meanwhile, the distal tentacle region, which is surely removed by such a cut, must be able to re-form during that time. The distal tentacle area is only beginning to be laid out by stage III, and can be duplicated in time for normal emergence.

There is never any region in which distal tentacles cannot form if enough time is allowed, as observed by Davidson and Berrill (1948), Peebles (1900), Driesch (1897), and during the course of these experiments. Peebles, using stems which had reconstituted to the stage in which both distal and proximal tentacle striations were present, made cuts through the proximal striations. The terminal segments never developed proximal tentacles, with one exception in more than 100 cases. Driesch, using stems of the same stage, made cuts midway between the two striated regions, and never observed proximal regeneration of the terminal piece, while the stem piece was always able to produce a complete hydranth. Finally, Gilchrist (1937) showed that a similar situation occurs in polyps of *Aurelia*. These have only a single tentacle group; however, fragments removed from the body of the polyp can always form the tentacles and a hypostome, whereas fragments of the hypostome, which is distal to the tentacles, cannot form tentacles unless some of the cells from the base of a tentacle are included. It may therefore be a rule that fragments from polyps and polyp primordia can reconstitute structures that are normally distal to the original location of the fragments, but not more proximal structures. Such a rule would not apply, however, to something like the gonophores of *Tubularia*, which Davidson and Berrill (1948) showed can form from a very broad region of the primordium.

By the time the pigment band of stage IV has formed the two bands of stage V, the region of proximal tentacle potency is restricted to the region occupied by the proximal band plus a part of the interband region, while a less sharp tendency toward the restriction of distal tentacle potency to the distal band is indicated. Figure 5 shows that segments that do not contain the distal band, or a part of it, tend not to form distal tentacles by the time of emergence, or else emerge with rudimentary distal tentacles.

When proximal tentacle striations have formed (stage VI), proximal tentacles apparently cannot form from any piece of the primordium distal to the striated region. Segments removed from between the pigment bands of this and earlier stages are of interest because they produce a spectrum of tentacle types. Evidently, the exact levels of the distal and proximal cuts determine whether the interband segments will be able to form distal or proximal tentacles, or both, or neither.

It is of interest to compare the extent of pigmentation with the extent of the regions in which tentacles can form, since these do not coincide in all stages. In stage II, no pigment concentration can be observed, and yet determination has already progressed to the point where proximal tentacles cannot form from the distal 0.5 to 1.0 mm. of stem. By stage III, the pigment ends about 3 mm. from the stem tip, and proximal tentacles can no longer develop from the terminal 2.5

to 3.0 mm. of stem. The region of proximal tentacle potency remains permanently in that position, while the limit of pigment moves distally, so that, by stage IV, it occupies only about the terminal 2.5 mm., and does not occur at all in the region in which the proximal tentacles are destined to form. In stage V, the pigmented region gives rise to two bands, with the proximal half of the pigment moving back so that it secondarily coincides with the region in which the proximal tentacles will develop. The earliest shrinking of the region in which proximal tentacles can form therefore appears to be one which limits the proximal tentacle potential to an unpigmented region.

Stevens (1901) has discussed the possible significance of the pigment in reconstitution, as a result of her studies of reconstituting *Tubularia* stems. The studies led her to conclude that the pigment is accumulated by the primordium from the circulating fluid of the gastrovascular cavity, into which it is released by breakdown of the endodermal ridges. She also stated that much of the pigment is expelled by the newly emerged hydranth (something not observed during these experiments) and, discussing the "formative substance" of Driesch and Loeb, said (p. 414), "The indication is that the red granules, far from being 'formative substance,' are in fact the only part of the cell material from the disintegrated endodermal ridges, which the animal cannot make use of in regenerating a hydranth, and therefore rejects at the first opportunity." Although there is no reason why a "formative substance" could not be ejected after it was no longer useful, it now appears that the pigment of *Tubularia*, at least in visible concentrations, does not contribute to reconstitution.

Cohen (1952) showed conclusively that some of the pigment that appears in the primordium is collected by the cells of the primordium from the circulating gastrovascular fluid, while Goldman (1953) showed equally conclusively that the rest of it is actually synthesized within the primordium. None of this information, of course, clarifies the role of the pigment in determination itself. However, as stated above, proximal tentacle determination can, and normally does, take place in the absence of discernible pigment. It is also significant that, during these experiments, occasional stems would never produce any visible pigment, even though they were perfectly able to reconstitute, producing colorless hydranths. Occasionally, the laboratory collectors brought in whole colonies of *Tubularia* in which very little pigment was discernible, and stems from those colonies reconstituted as well as stems from deeply pigmented colonies. The conclusion therefore follows that the concentrated pigment that accumulates during reconstitution is a secondary result, and not a cause, of tentacle determination.

Summary and Conclusions

The primordia from five stages in reconstitution of *Tubularia crocea* were cut into short segments, using the distribution of pigment as a landmark, and the segments were allowed to develop in isolation from the rest of the primordia. When the differentiated segments were ready to emerge, the completeness of reconstitution was observed. It was hoped in this way to determine what relationship exists between the characteristic pink pigment that accumulates during reconstitution, and distal and proximal tentacle formation, and, also to learn something

of the time sequence and extent of determination. The following conclusions are based on the experiments.

1. Stem segments less than one millimeter in length tend to form undifferentiated clumps of cells, or bipolar hydranths which have two sets of distal tentacles and may or may not have proximal tentacles. Unipolar hydranths (one set of distal tentacles and no proximals) are rare.

2. Two hours after decapitation, the distal millimeter of stem can no longer form proximal tentacles, but distal tentacles can form from any fragment of appreciable size.

3. At stage III, in which the pigment is vaguely concentrated in about the distal three millimeters of stem, proximal tentacles cannot form in fragments from the distal 2.5 to 3.0 mm., and distal tentacle formation shows a tendency to occur less frequently in the proximal half of the pigmented area than in the distal half.

4. At stage IV, when the pigment is sharply confined to about the distal 2.5 mm. of stem, the region of proximal tentacle potency occupies the same region as in stage III, which, in stage IV, is no longer pigmented.

5. At stage V, in which two pigment bands exist, the distal band appears necessary for the formation of distal tentacles in time for emergence, while the proximal band represents the only region that regularly can give rise to proximal tentacles.

6. At stage VI, in which the proximal pigment band is striated, there is an increased tendency toward the restriction of distal tentacle potency to the distal band.

7. The earliest disappearance of the ability to form proximal tentacles occurs in the pigmented region, when the proximal tentacles can form only in regions lacking visible pigment.

8. The pigment of *Tubularia* appears to play no causal role in the progressive disappearance of regenerative ability. It seems most likely, therefore, to be a by-product of the activity that accompanies the reconstitutive process.

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INFECTION OF COCKROACHES WITH HERPOMYCES (LABOULBENIALES). I. LIFE HISTORY STUDIES[1, 2]

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Largely by chance it was discovered that our laboratory colony of the oriental cockroach (*Blatta orientalis* L.) was infected with *Herpomyces stylopygae* Spegazzini. Subsequently, some of our boxes of the german cockroach (*Blattella germanica* L.) were found infected with *H. ectobiae* Thaxter. How long these infections have been present in our colonies is not known. More recently we introduced a colony of a giant tropical cockroach (*Blaberus craniifer* Burm.) from Key West, Florida; on arrival these specimens were found infected with *H. tricuspidatus* Thaxter. Since most past studies of the Laboulbeniales, and all of those on cockroach-inhabiting species, have been by taxonomic mycologists, we decided to examine the etiology and the host-parasite relationship from an entomological point of view. Incidentally, some points of strictly mycological interest were noted and will be recorded.

Although we have utilized all three of the above species of *Herpomyces*, the majority of our data deal with *H. stylopygae* on oriental cockroaches. The data are sufficiently voluminous that they are being presented as a series of papers. In the present paper we are presenting data on the life history of the fungus, its transmission, and some notes on the structure of asci and ascospores. In another paper (Richards and Smith, 1955a) we are presenting our histological and histopathological work. A separate note (Richards, 1954) treats histochemical differentiation of the fungal walls. Subsequent papers are planned covering our experimental work on host specificity, location on the host specificity, germination and development.

Due to the tremendous variability within the Laboulbeniales, one should remember that all of our data deal with the single genus *Herpomyces* found only on cockroaches, and most of our data deal with the single species *H. stylopygae*. The majority of the approximately 1500 described species (Benjamin and Shanor, 1950) of Laboulbeniales occur on species of Diptera and Coleoptera (flies and beetles). Morphology and habits are quite diverse. For instance, there are a number of records of spores germinating while still within the perithecium

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(*Laboulbenia, Dioicomyces, Dimorphomyces,* etc.); with thousands of specimens examined we have never seen this phenomenon in *Herpomyces*. Generalization seems dangerous at the present stage of knowledge of this group.

The Validity of Herpomyces stylopygae Spegazzini

Thaxter (1908) described the species *H. periplanetae* as occurring on both the american (*Periplaneta americana* L.) and the oriental (*Blatta, = Stylopyga, orientalis* L.) cockroach. Subsequently, Spegazzini (1917) separated the form found on the oriental cockroach on the basis of relatively slight differences: larger perithecia, a blunter shield over the secondary receptacle, a blackened base to this shield (Fig. 3) and a few other minor points. Thaxter (1931) questioned this separation on such slight differences, but we have had to conclude that the two are distinct (whether species or strains) because we have been unable to obtain development of the form from oriental cockroaches on *Periplaneta americana, P. australasiae* or *P. brunnea,* the listed hosts of *H. periplanetae* (details will be given in Richards and Smith, 1955b). Yet there is no difficulty in transferring the infection in the laboratory from one oriental cockroach to another.

Distribution of H. stylopygae on Blatta orientalis

The infection is most commonly limited to the antennae (Fig. 1). Of 50 infected cockroaches taken at random from the colony and examined carefully, 38 (75%) had plants only on the antennae, 11 (22%) had heavy antennal infections plus some plants on the maxillary palpi, and a single specimen (2%) had a heavy antennal infection plus some plants on both the maxillary and labial palpi. No plants were found elsewhere on the body. However, with hundreds of roaches handled (but not searched) subsequently we have found several males with a few plants on the cerci, and one male with four plants growing close to one another on the ventral surface of the right metathoracic femur. In no case have plants been found elsewhere on the body except when there was a very heavy infection on the antennae. And nowhere on the body except on the antennae do the infections become so heavy they almost hide the surface of the infected part. The antenna shown in Figure 1 represents only a moderate infection; heavy infections so cover the antennae that they photograph less well.

The long antennae of cockroaches are their investigative organs. In a colony, and in nature, they are waved about in all directions making repeated contact with their substrate and their neighbors. When under crowded conditions, common in nature as well as in culture boxes, cockroaches are continually making contact with the antennae of other individuals. The contact involves all exposed surfaces, even the ventral surface, as one cockroach runs over another. As shown in Figures 1 and 2, mature perithecia commonly have a spore or spore group protruding from the subterminal aperture for 80–85% of its length. Such spores are dislodged by a touch or jarring. Considering the habits of cockroaches and the presence of such easily dislodged spores, it is obvious that the antennae must serve as efficient spore brushes and spore dusters. Further, it seems obvious that under crowded conditions spores should become placed on all portions of the body. It is simple to demonstrate that this is indeed true; areas of the cockroach body wall

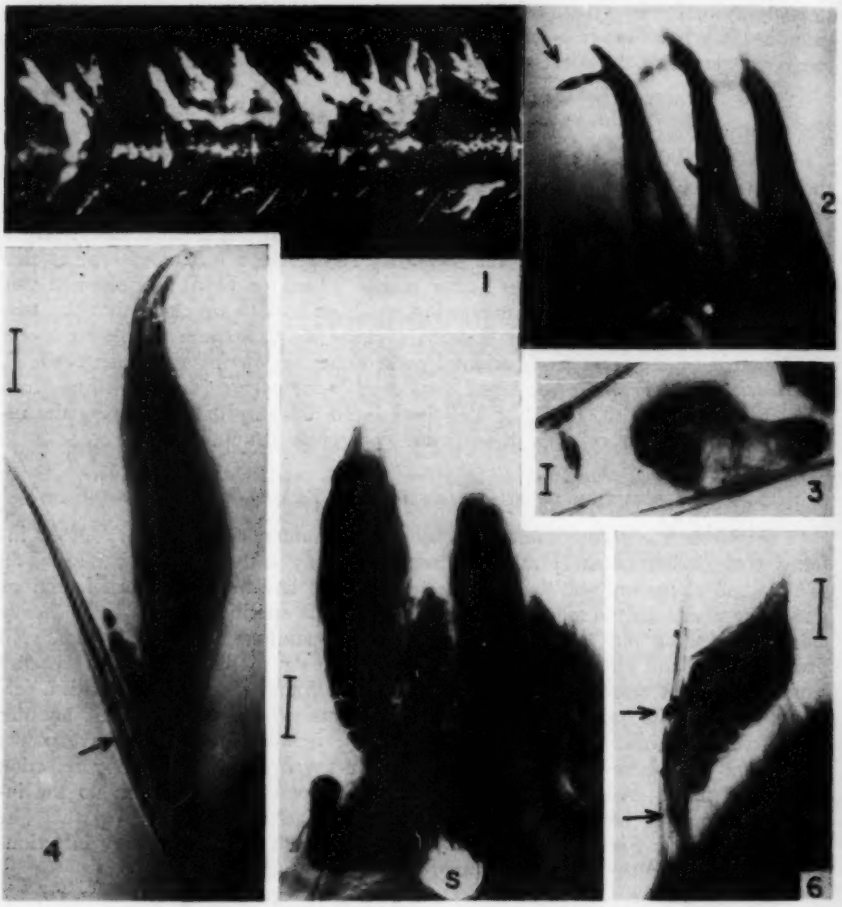

FIGURE 1. A small portion of an antenna of *Blatta orientalis* moderately infected with *Herpomyces stylopygae* (living). There are fifteen plants on these six antennal segments. Note spores protruding from the subterminal apertures of many of the perithecia.

FIGURE 2. Higher magnification of mature, living perithecia showing a spore or pair of spores (arrow) protruding from each perithecium (only one in clear focus).

FIGURE 3. Basal shield of *H. stylopygae* showing naturally blackened base and stained apex (cotton-blue). Note also pair of spores on cuticle surface (one in 2-cell stage, other in 4-cell stage. Bar 10 μ.

FIGURE 4. Young mature female plant of *H. ectobiae* on antennal seta of *Blattella germanica*. Note basal holdfast of spore (arrow) and basal prolongation of plant to setal base. Cotton-blue stain. Bar 10 μ.

FIGURE 5. Larger female plant of *H. ectobiae* spreading across antennal surface of *B. germanica* after reaching base of seta (S). Cotton-blue stain. Bar 10 μ.

FIGURE 6. Young male plant of *H. ectobiae* on antennal seta of *B. germanica*. Note spore in 4-cell stage alongside this plant on seta (holdfasts indicated by arrows). Cotton-blue stain. Bar 10 μ.

excised, stained with cotton-blue in lacto-phenol and examined under the microscope show spores all over the body surface. Clearly the hypothesis that the specific locations of infections are due to transfer of spores to only those locations, as suggested by Thaxter (1896) and preferred by Benjamin and Shanor (1952) for *Laboulbenia* species, cannot hold for infections of *H. stylopygae*. We hope at a later date to discuss this question of development to maturity only on certain areas, but we might mention here, in passing, that in single insect species histochemical differences in the cuticle surface for different areas of the body have recently been reported (Richards, 1952).

At frequent intervals, cockroaches clean their antennae by bending them down and pulling the shaft of the antenna through the mouth parts, thus masticating and scraping them. Likely this is the source of infections developing on palpi though infection from another individual does not seem impossible. It is tempting to speculate that the lower incidence of infection on palpi may be due to the antennal infection having to mature first but the very low incidence on all parts of the body except the antennae makes such a speculation decidedly questionable.

On the antennae plants may be located anywhere but are seldom seen on the most basal segments. On the average there are about one hundred segments in each antenna of *B. orientalis*. Approximately equal numbers of plants grow to maturity on segments of the apical 90% of the antennae, more local distribution in individual cases being shown by actual counts of spores and plants to be correlated with and hence due to corresponding irregularities in the chance distribution of spores.

Plants may be found growing on hard sclerite areas or on soft membrane. Correlated with the fact that most of the surface of the antenna is sclerotized, most of the plants are found on sclerites. Commonly they are associated with setae and penetrate through the setal sockets but also, commonly, they penetrate through sclerite where no seta is located (see Richards and Smith, 1955a).

Infections develop much more heavily on males than on females. Rough estimates from 24 ♂ and 24 ♀ taken from infected colonies show that there are on the average more than twice as many plants on infected males. Infections on nymphs are never very heavy but infected individuals of all instars can be found. In a specific test where 4 ♂, 4 ♀ and 4 nymphs were exposed to infection from 8 adult males (in a pint jar) for just one day and then isolated, 117 mature plants were found on the males, 62 on the females but only 11 on the nymphs at about two weeks later. For these counts, both antennae were amputated at the base, stained with cotton-blue in lacto-phenol and the number of spores and plants tabulated for every antennal segment. It was found that there were 416 spores or plants on the females and 525 on the males, *i.e.*, about 50 per antenna, but only 119, *i.e.*, 15 per antenna, on the nymphs. Since infection was from adult males, it follows that infected males distribute approximately equal numbers of spores to adults of either sex but significantly less to nymphs. Of more interest is the fact that exposure to infection was on a known single day and examination was made at a known subsequent interval (11–14 days). If one assumes all spores will germinate and grow on an antenna, all of these should have been reaching maturity at the time of examination. Tabulation was made under four headings: 2-cell spores, 4-cell spores, immature plants, and mature plants. Of the total of 1060 spores, only 16 were still in the 2-cell stage, but 502 had not progressed past

the 4-cell stage. This growth failure of almost 50% was not uniformly distributed. Tabulating the values:

on nymphs (119 spores): 29% past 4-cell stage, 9% mature, at 13–14 days,
on females (416 spores): 44% past 4-cell stage, 15% mature, at 12–13 days,
on males (525 spores): 67% past 4-cell stage, 22% mature, at 11–12 days.

Clearly, either the per cent growing on males > on females > on nymphs, or there is a delay at the 4-cell stage with sporadic initiation of subsequent development being most frequent on males and least frequent on nymphs. At this 11–14 day time almost exactly one-third of those which had passed the 4-cell stage had mature perithecia in each group. Perhaps this can be interpreted as favoring the second alternative. Such a difference in frequency of initiation of later development would give the observed point that, both in colonies and after known infections, about twice as many *mature* plants are seen on males as on females.

We have succeeded in inducing growth of *H. stylopygae* only on live cockroaches. Plants will not grow past an early germination stage on exuviae (shed skins) or on excised and explanted pieces of abdominal cuticle or integument floating on broth in a humid atmosphere; they will not grow on amputated antennae even at 100% relative humidity; and if the cockroach dies the plants wither within a few days. However, plants are active for some time after infected antennae are amputated. An amputated antenna makes a good spore brush for experimental infections. After one use it may be held for a few hours in moist air and used again. Actually this is illustrated by Figure 1. In mounting this amputated antenna for photographing, all the spores were dislodged; the mount was held in moist air and this photograph taken some hours later.

Following infection with spores, mature *H. stylopygae* are found on *Blatta orientalis* in a little less than two weeks (25–27° C.). About the same length of time is required for *H. ectobiae* on *Blattella germanica*. Somewhat longer, perhaps three weeks, is required for *H. tricuspidatus* on *Blaberus*.

Egg capsules from heavily infected *B. orientalis* females give batches of nymphs which do not develop infection. Also spores dusted or brushed onto egg capsules or exposed eggs do not develop into visible plants. Finally, surgical implantation of infected antennal fragments into various parts of the body cavity of oriental cockroaches gave entirely negative results.

In conclusion, infection is readily obtained by direct contact placing the spores on an appropriate external surface but, in our experience, by no other means. It is reasonable to suppose, as Thaxter (1896) did, that infection might also be indirect by deposition of a spore on any surface from which another insect by chance collects it onto itself. Lindroth (1948) has recently reported such indirect transmission in certain *Laboulbenia* spp. where apparently spores may survive in soil for some weeks before being picked up by a passing beetle. We have not attempted to check this possibility but to judge from our experience with glass needles (Table I) we would suspect that while many spores are doubtless dropped, few would ever subsequently become transferred to a passing insect.

Notes on Asci and Spores of H. stylopygae

Having only dry material at his disposal, Thaxter was unable to be sure how many spores were in an ascus. Most Laboulbeniales have four spores per ascus

but some have eight. Clearly there are eight in *H. stylopygae* (Figs. 7 and 8). The asci are a fat cigar-shape and measure about 12 × 60 μ, those used for the photographs seeming to be broader because somewhat flattened by cover-glass pressure to bring the spores into one focal plane.

An apparently new point is that in this species the asci do not disintegrate to liberate spores within the perithecia. Perithecia of living plants teased in a drop of water liberate both spore-filled and empty asci (Figs. 8 and 9). The spore-filled asci were sometimes seen to liberate their spores one after another through

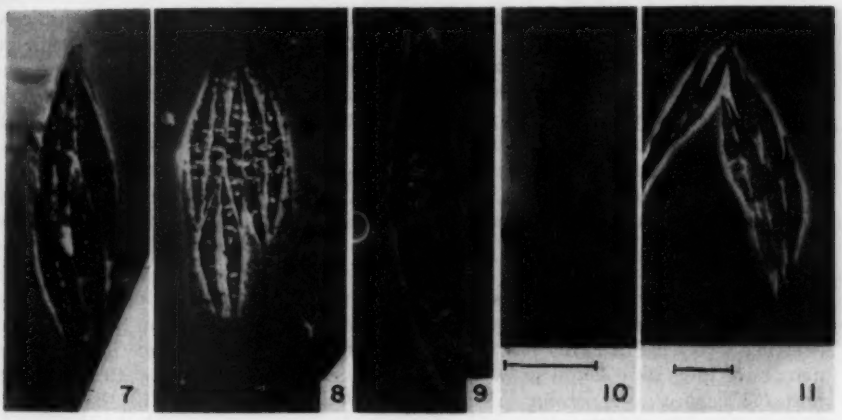

FIGURE 7. Mature ascus with its eight spores after staining with the gram stain. From perithecium of *H. stylopygae*. Note that both spores and the ascus wall are gram positive. Bar 10 μ.

FIGURE 8. Mature ascus of *H. stylopygae* in water; somewhat compressed by coverglass pressure. Dark phase-contrast photomicrograph of living material. The pressure used to get all the spores into one focal plane has resulted in a rupture of the ascus wall and extrusion of material at the lower right of the figure (such rupture is not normal). Bar 10 μ.

FIGURE 9. Empty ascus or ascus ghost of *H. stylopygae* in water. Dark phase-contrast photomicrograph (not stained). Note contents, absence of any indication of rupturing or disintegration (spores released through terminal pore), absence of any operculum, and apparently unitunicate condition. Bar 10 μ.

FIGURE 10. Electron micrograph of empty ascus ghost of *H. stylopygae*. Shows extreme thinness of dry ascus wall, and hence small amount of solid material therein, and no indication of more than a single layer. Bar 10 μ.

FIGURE 11. Living spores of *H. stylopygae* in water; teased from fresh perithecium. Note clustering of the spores. Bar 10 μ.

an aperture in the end as drying of the water began to exert cover-glass pressure on the ascus. No rupture of the ascus wall was evident, and the terminal hole is not visible after the spores have been discharged (Fig. 9). Although the observed liberation of spores was under unnatural condition, the presence of numerous empty asci within partially emptied perithecia implies that liberation of the spores is normally via a perforation (presumably at the distal end) rather than by disintegration. As Figures 9 and 10 show, the asci are inoperculate and unitunicate (see Luttrell, 1951).

The spores seem to be always liberated from the asci while within the perithecia. At least we have never seen asci ejected, the canal of the perithecium is too small for an intact ascus, unless the ascus were compressed, and we obtained only spores, never asci, in picking spore groups off the tip of the perithecium (Table I).

After liberation of the spores, the empty ascus is left as a ghost containing many highly refringent granules and droplets (Fig. 9). Commonly, active Brownian movement is evident. Dr. Benjamin suggests that these granules and droplets represent epiplasm, *i.e.*, protoplasm remaining after the aggregation of most of the material into spores (by "free cell formation"). The interior of mature perithecia contains gram-negative, bipolar-staining, short-rod, bacteria. In stained smears these are particularly abundant around empty or torn asci.

The ascus wall, like the spores, is gram-positive, and of the order of 0.1 μ thick. On electron microscope grids the empty asci dry down to give ghosts of about 9×43 μ; correcting for the flattening this gives about 6×40 μ. The electron micrographs show only that the ascus wall is exceedingly thin after drying, not over a few hundredths of a micron thick, and that it is heavily contaminated with debris, presumably from the granule-containing fluid within the empty ascus (Fig. 10).

It is generally stated that spores are discharged in pairs throughout the Laboulbeniales although various published drawings of asci do not show such association of spores in pairs. Dr. R. K. Benjamin tells us that there is good evidence to believe that this generality holds for many genera because the dioecious plants are invariably found growing in pairs even though widely scattered on the host (*Dioicomyces, Aporomyces, etc.*).[3] However, Thaxter (1931) has already remarked on the commonness of single plants in *Herpomyces*, and it became evident early in our studies that this was partly or perhaps entirely due to the discharge of single spores. Single plants could also originate from the loss of one member of a spore pair; we do not know whether this occurs naturally but in lacto-phenol mounts one sometimes sees two sets of holdfasts but only a single spore (Fig. 13), indicating the loss of one member of the pair either before or during preparation of the slide.

To determine the actual number of spores discharged at one time, a census was made of the spore clusters protruding from the perithecia of 128 ($= 2^7$) plants. For this purpose, infected oriental cockroaches were observed under high power of a dissecting binocular microscope, protruding spore groups picked off individual perithecia by touching the group with the tip of a fine glass microneedle, and then transferring the spore group to a droplet of cotton-blue stain in lacto-phenol. Subsequently the coverglasses bearing rows of stain droplets were inverted on a depression slide and each droplet carefully searched, with the final check on spore count being made under oil immersion. Validation of the technique was observational: the protruding spore groups could be seen to be removed;

[3] This opinion assumes that all spores germinate and grow into readily located plants. An alternative possibility is that plants mature only when in pairs. Some reason for holding the second possibility in mind until it is disproven comes from data on the germination of *Herpomyces* on hosts on which they will not mature. These data will be presented subsequently. Also we have occasionally seen single plants of *H. ectobiae* alongside an ungerminated spore; these could have arisen from growth of only one of a spore pair (Fig. 6).

they could be seen on the microneedle, and the microneedle was clean following immersion in the stain droplet.

The data may be tabulated in various ways (Table I). There is some question as to whether these particular numbers could be reproduced because the one-two dozen cockroaches used were not truly randomized and we noted that one individual would yield only single and paired spores whereas the next one might give a high percentage of multiple groups. Perhaps such individual differences are due to the age of the plant or (more likely, we think) due to the length of the time interval since protruding spores were last brushed off. However, the important points are independent of the above uncertainty. These are:

(1) The spore groups protruding from perithecia may be sufficiently adherent to remain together as a compact cluster in the stain droplet, or they may dissociate into several (up to 4) smaller groups, even to single spores.[4] In column

TABLE I

Tabulation of data from 128 protruding spore groups picked off the tips of perithecia (columns 1 plus 2), and from the 188 spore groups into which these separated in the stain droplets (columns 5 plus 6). Total number of spores 362

Number of spores per group	From 128 perithecia		Total spores per group		From 188 adhering groups		Total spores per group	
	Number	Per cent	Number	Per cent	Number	Per cent	Number	Per cent
1	14	11	14	4	75	40	75	21
2	62	48	124	34	82	44	164	45
3	10	8	30	8	10	5	30	8
4	32	25	128	35	16	9	64	18
5	5	4	25	7	3	1.6	15	4
6	1	0.8	6	1.7	1	0.5	6	1.7
7	1	0.8	7	2	0	0	0	0
8	2	1.6	16	4.4	1	0.5	8	2.2
12	1	0.8	12	3.3	0	0	0	0

1, the numbers found per stain droplet (= per perithecium) are listed; in column 5 the numbers found per association group are listed. The 128 perithecia actually gave 188 groups in the stain. Perhaps a similar dissociation could occur on transference to the surface of another host (see below).

(2) The ejection of single spores is common.

(3) Most of the spore groups consisted of 1–4 spores but higher numbers up to 12 were occasionally found.

(4) The values shown in columns 1–4 of the table do not represent any common form of distribution; they are probably the product of several ejections preceding dislocation of the group (see below). In contrast, the values in columns 5–6 (listings for 188 adhering groups) do represent a skew-form distribution

[4] Sets of two were usually pairs but commonly two singles; sets of three were either 3 or $2+1$ or $1+1+1$; sets of four were 50% tetrads but sometimes $2+2$ or less often $2+1+1$ or $1+1+1+1$; sets of five consisted of 5 or $3+1+1$ or $2+2+1$ or $2+1+1+1$; the set of six consisted of $4+2$; the set of seven was $4+2+1$; the sets of eight were one set of 8 and one of $3+2+2+1$; and the set of twelve was $6+5+1$.

curve commonly encountered in biology; they also give a closer approximation to what one can see on the surface of cockroaches from infected colonies.

Another important point not shown by the table is that the aperture in the perithecium is not of sufficient diameter to permit the simultaneous passage of groups of 8 or 12 spores, and is questionably adequate for the simultaneous passage of a tetrad. Probably the answer is to be deduced from the observation that perithecia under coverglass pressure in a drop of water may be seen to eject spores like bullets coming out of a machine gun. They are ejected too rapidly for accurate determination of how many emerge at once but under these conditions they seem to come out singly. The important point, however, is that they are all ejected for about the same distance and hence align to form a cluster which to visual inspection may appear to be as tight a pack of adhering spores as if they had been ejected

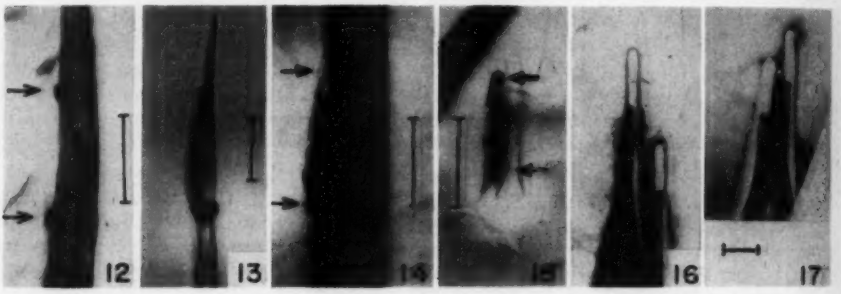

FIGURE 12. Part of a setal shaft from antenna of *Blatta orientalis* showing the two spore holdfasts (arrows) of *H. stylopygae* after dislodgment of the spore during preparation of slide. Cotton-blue stain. Bar 10 μ.

FIGURE 13. Spore of *H. ectobiae* in 4-cell stage on antennal seta of *Blattella germanica*. Note that there is a pair of basal holdfasts; presumably one spore of a pair became dislodged. Cotton-blue stain. Bar 10 μ.

FIGURE 14. Spore of *H. stylopygae* in 4-cell stage on an antennal seta of *Blatta orientalis*. Shows basal and apical holdfasts (arrows) but the sheath is almost invisible. Bar 10 μ.

FIGURE 15. Germinating spore of *H. stylopygae* on surface of antenna of *Blatta orientalis*. Note characteristic method of growing over the basal holdfast. Cotton-blue stain. Bar 10 μ.

FIGURES 16 AND 17. Electron micrograph of ends of two spores of *H. stylopygae*. The line in the underlying formvar membrane shows the original position and shape of the end of the spores. Under electron bombardment the spores have shrunk back and developed blunt ends (opaque). Bar 1 μ.

as a group. We suspect that ejection from the perithecia of *H. stylopygae* is of single spores and that groups of pairs or more represent two or more ejections which adhere because of the sticky external coat (said to be "gelatinous").

Examination of amputated antennae or other areas of the body wall, or of shed skins (exuvia), stained and cleared with cotton-blue in lacto-phenol and examined as whole mounts show large numbers of single and paired spores both on setae (Figs. 13–14) and on the body wall proper (Figs. 3 and 15). Larger groups are uncommon but occasionally seen. These data suggest that the numerous single plants arise from the development of single spores, and that at least some of the large plant clusters commonly seen arise from the development of multiple spore clusters.

Incidentally, both male and female plants develop to maturity in isolation; presumably the crowding permits fertilization of such female plants by nearby males. Single and paired spores (Fig. 13) as well as single (Figs. 4 and 6) and paired (Fig. 5) plants were also found with *H. ectobiae*.

Spores are normally and probably always discharged in the 2-cell stage. All of the 362 spores of Table I were 2-celled. Suspended in distilled water (Fig. 11) they measure 2–3 × 20–30 μ. Likely these spores are somewhat enlarged by uptake of water. A dried spore examined in the electron microscope measured 1.4 × 25 μ with the bluntly rounded tips (Figs. 16–17) measuring 0.3 × 2.5 μ. These spores are colorless when ejected and have a thin wall. Both nuclei and cytoplasm are acidophilic in staining and gram-positive (Fig. 7). In the living state the clear nuclei are about 2 μ in diameter and are surrounded by small granules a small fraction of a micron in diameter (Fig. 11). The birefringent walls of the spore show in electron micrographs microfibers a few hundreths of a micron in diameter—a common microfiber diameter for chitinous membranes (Richards, 1951).

On the host the spores quickly become 4-celled and then usually show a brown or black spot at each end (Figs. 12–15). There is little or no visible difference between the two ends of the spores in either the 2-cell or 4-cell stages. That the brown or black spots at each tip serve as holdfasts, not previously reported to be present in any species of Laboulbeniales, is shown by the fact that when a spore becomes dislodged during preparation of a lacto-phenol mount these blobs may remain stuck firmly to the body wall or seta (Fig. 12). In the latter part of the 4-cell stage the germinating spores do show a longitudinal differentiation (Fig. 15).

Quite possibly the spores always emerge from the perithecium in one orientation but it does not seem reasonable to assume that with a brushing or dusting dissemination they always become applied to setae or the general body wall in one particular orientation. On setae they are always parallel to the longitudinal axis of the seta, as would be dictated by the action of surface forces, but on the general body wall they are oriented in all directions parallel to the surface. Yet on setae the plants always develop with the base at the proximal end of the seta, and on the body wall the apices of the plant project distally in relation to the antenna. It seems necessary to assume that either the orientation of the developing plant is somehow imposed by its position on the insect's cuticle, or that only those plants appropriately oriented develop (those in reverse orientation not developing and so contributing to the number of ungerminated 4-cell spores always present). Incidentally, artificially germinated spores also show visible differentiation of the two ends shortly after the 4-cell stage, as will be treated in a subsequent paper.

Volume Increase During Growth of H. stylopygae

Assigning appropriate geometric figures to the various stages one can calculate approximate volumes and hence volume increase during growth. Cigar-shaped spores, 2 × 25 μ, have a volume of approximately 25 cubic microns. Mature female plants calculated as a combination of cones, cylinders and elliptical spheroids have a volume 90,000 to 400,000 cubic microns, depending primarily on the number and size of the perithecia. The most gigantic female plants with haustorial bulbs 40 × 100 μ would have a volume of approximately 500,000 cubic

microns. The portion of the plant visible on the surface of the insect, *i.e.*, plant minus haustorium, is considerably smaller, averaging 75,000 to 350,000 cubic microns. (Details of the structure of haustoria are given in part 2 of this series.)

The volume of a female plant is, then, 3500 to 20,000 times that of the spore from which it grows. Male plants are much smaller. Rough calculations from illustrations and measurements given in Thaxter's monographs show that other species of Laboulbeniales increase by 300 × to > 100,000 × the spore size (not including the volume of haustoria presumably present). Obviously a considerable amount of nourishment must be obtained from the host even if we assume that the average density of the spore is considerably greater than that of the mature plant.

Loss of the Infection During Molting

In most cases (Diptera, Coleoptera, Hymenoptera), Laboulbeniales have been described from adult insects which do not molt. But in cockroaches they are found both on adults and the similar-appearing cohabiting nymphs. In adults the infections seem to survive until the insect dies, and in some cases Thaxter has described what he considered to be age changes in certain species. In nymphs of both *Blatta* and *Blattella* the infection is completely lost at ecdysis (molting). The external portions of the plant are visible on the exuvia (shed skin) and appear normal until they dry up.

In a direct test, 18 nymphs of *B. orientalis* were caught in the act of molting. The exuviae were checked and the infections mapped. The individuals were isolated and checked at intervals for 22 days (> 50% longer than the usual life cycle of the fungus). In no case did any infection develop. Many more have been tested indirectly by our finding that the most convenient method for getting known-to-be-uninfected cockroaches is to segregate into a clean container specimens by chance caught molting in our cultures. To the best of our knowledge this phenomenon of the complete loss of infection by molting has not previously been reported.

The failure to regenerate an infection after the thalli have been lost at molting shows that the large haustorial bulbs (see part 2) which must be left behind in the cockroach's epidermis cannot regenerate the lost plants. We are inclined to correlate this with the absence of nuclei from the haustoria but obviously other factors could be involved.

We have never seen any indication of the development of resistance to infection. Infected individuals freed of their infection by molting seem to be just as readily infected as ones not previously exposed to the fungus. In fact, in heavily infested colonies, most of the individuals have been re-infected in each instar and yet the really heavy infections develop on adults. Also, the re-infection may occur immediately because we have obtained some infected individuals after segregating specimens of *B. orientalis* which had molted so recently that their cuticles were still white (*i.e.*, < one hour after molting).

Notes on H. ectobiae Thaxter

H. ectobiae growing on *Blattella* (= *Ectobia*) *germanica* has not been studied as intensively. Some differences from the habits of *H. stylopygae* have been noted.

Infections with *H. ectobiae* seem always to start on setae (Figs. 4–6). Usually they grow to the setal base and make an additional haustorial penetration there (Figs. 4–5). When they spread across the antennal surface larger plants result (Fig. 5). Of the hundreds we have examined no one has been seen in such a position that it could not have started development on a seta. We have noticed that male plants are more likely to reach large size without attaining the setal base than female plants are (Fig. 6). Female plants seem always to attain the setal base prior to maturing perithecia (Figs. 4–5).

Unlike *H. stylopygae, H. ectobiae* develops on any part of the surface of its host though nowhere attaining the density of heavy infections on antennae of *Blatta* and *Blaberus*. There is no evident pattern to the infection and no obvious differences correlated with sex of the host. Systematic survey with tabulation of infected areas on 34 individuals of *B. germanica* showed mature plants on antennae, maxillary and labial palpi, frons, coxae, femora, tibiae and tarsi of all pairs of legs, wings, thorax, abdomen (dorsal, lateral and ventral), and cerci. Some individuals had plants growing simultaneously on most of the above regions. Nymphs of *Blattella* commonly develop heavier infections than nymphs of *Blatta* do.

The spores of *H. ectobiae* regularly have a brown or black spot at each end in both the 2-cell and the 4-cell stage (Fig. 13); at least the proximal one of these appears to serve as a holdfast.

Summary

1. *Herpomyces stylopygae* Speg. is shown to be distinct from *H. periplanetae* Th. by host specificity tests.

2. Spores of *H. stylopygae* are found all over the surface of oriental cockroaches but mature plants are mostly found on the antennae, seldom on palpi and only rarely elsewhere. They grow on setae or on hard or soft cuticle but only on a living cockroach. Infections are heavier on males and on adults and experiments show the infection is disseminated by contact.

3. The ascus contains 8 spores which it liberates within the perithecium through a terminal perforation, leaving the ascus as a fluid-filled ghost.

4. Spores are ejected from the perithecia in various numbers, not just in pairs (Table I). Mostly the groups protruding from the subterminal apertures of the perithecia (Fig. 2) consist of 1–4 spores but groups as large as 12 spores were found. The presence of single, paired and multiple spore groups protruding from perithecia and found on the surface of hosts is correlated with the presence of single, paired and multiple plants on infected cockroaches.

5. Antennae of infected cockroaches serve as efficient spore brushes and dusters.

6. Spores become firmly attached to the cockroach's cuticle by holdfasts developed at both ends.

7. Development from spore to mature perithecia takes nearly two weeks.

8. The volume of a female plant is 3500–20,000 times that of a spore. So much material cannot be obtained from a minute volume of cuticle. A tubular haustorium through the cockroach's cuticle was found to expand into a large bulb in the epidermal cell layer.

9. Infections on adults persist but infections on nymphs were found to be completely lost when the nymph molts. The fungus plants are found intact on the shed skin.

10. There appears to be no development of resistance since individuals freed of infection by molting can be readily reinfected.

11. Some notes are given on spore structure, and on differences shown by *H. ectobiae*.

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CELL MOVEMENT, RATE OF REGENERATION, AND THE AXIAL GRADIENT IN TUBULARIA [1]

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One of the great unifying concepts in the field of development has been that of the axial gradient. As Child envisions the development of polarity in an organism, there is a quantitative gradient in metabolic rate which marks the primary axis, this gradient being responsible for the establishment of qualitative differences along the axis during the course of development (Child, 1941). Such gradients have been demonstrated in a wide variety of plants and animals, among which is the extensively investigated hydroid, *Tubularia*. In this animal the axial gradient is manifested physiologically by a decreasing rate of hydranth regeneration as one proceeds from distal (apical) toward proximal (basal) levels of the hydrocaulus or "stem." This phenomenon was first observed by Loeb (1892), who did not consider the differences in rate to be significant, but confirmation by a host of other workers (Driesch, 1899; Morgan, 1905; Child, 1907a; Hyman, 1920; Barth, 1938, 1940; Spratt, unpublished) leaves no doubt concerning its reality.

During a recent investigation of the mechanism of physiological dominance in *Tubularia* (Steinberg, 1954) it became necessary to measure the rates of regeneration of distal and proximal ends of stems ligated in the middle. In such stems the distal end regenerates considerably faster than the proximal end (Morgan and Stevens, 1904; Peebles, 1931; Barth, 1938). It was observed that once the primordium of the regenerating hydranth was established as a thickened zone near the cut end, differentiation seemed to proceed at about the same rate at both the distal and the proximal ends. The increased time for proximal regeneration apparently was due to a delay in the establishment of the primordium rather than to a slower rate of its differentiation into a fully formed hydranth. Since the experiment was set up with another purpose in mind, the measurements necessary to test the validity of this observation were not made, but other considerations seemed to lend weight to this view.

The axial gradient in *Tubularia* is manifested not only by differences in rate of regeneration, but also by a gradual diminution in size of the primordium formed as pieces are taken from increasingly basal regions of the stem (Driesch, 1899; Hyman, 1926; Barth, 1938). There is also a direct correlation between the length of a piece and the size of the primordium which it develops (Driesch, 1899; Child, 1907b; Peebles, 1931; Spratt, unpublished), contrary to the results of Hyman (1926), longer pieces giving rise to larger primordia. It was found by Peebles (1931) that regeneration could be delayed and the size of the as yet unformed

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primordium of a long stem reduced by removing a large piece from the proximal end of the stem only if the piece was removed before 13 hours of the regeneration period had elapsed. These observations were confirmed by the author (Steinberg, 1954), in whose material, however, a period of 8 hours sufficed to produce the effects obtained by Peebles only after 13 hours. It was shown in addition that the period preceding the appearance of the primordium is one during which the tissue shifts toward the distal end of the perisarc, providing additional cells for incorporation into the primordium, and that rate of regeneration and the size of the regenerant are both functions of the number of cells available. By these experiments it is shown that both rate of regeneration and the size of the regenerant are fixed before the primordium appears. Certain factors, such as the tissue movement, which affect both of these parameters of regeneration, appear to operate exclusively before primordium formation. The evidence presented above applies to systemic factors which affect the rate of regeneration and the size of the primordium at a given level of the stem. It does not, however, exclude the possibility that the rate of differentiation of the primordium into a completed hydranth may vary from level to level or as a function of a large number of other natural variables affecting regeneration rate.

Rate of regeneration in *Tubularia* varies as a function of the following systemic parameters: length of the piece, width of the piece (Hyman, 1926), apico-basal level from which the piece is taken, and distal versus proximal regeneration site on the piece. These variables were chosen for investigation in the present study, the purpose of which was to ascertain whether the differences in rate of regeneration which exist between long versus short stems, narrow versus wide stems, the distal versus the proximal ends of ligated stems, and finally apical versus basal stem levels all lie in the period of tissue movement, or whether under the various conditions mentioned the differentiation process itself proceeds at different rates.

Materials and Methods

Straight, clean, unbranched stems from freshly collected colonies of *Tubularia crocea* were used exclusively. The proximal cut was always made slightly oblique in order to distinguish between distal and proximal ends. Regenerating stems were kept individually in labelled stender dishes of two-inch outside diameter containing ⅜ of an inch of sea water and maintained at a fairly constant temperature on a sea water table over which fresh sea water was constantly flowing. The time of cutting of each stem was noted, and at intervals of a few hours until regeneration in all groups was complete, each stem was inspected and its regeneration stage and the time recorded. To maintain objectivity, the previous history of a stem was never consulted in making a new recording of its regeneration stage.

The animals were divided into 5 pairs of groups as follows, with 20 stems in each group.

I (20° C.)
- Group A. 8-mm. stems with the distal end taken two mm. below the hydranth.
- Group B. 8-mm. stems with the distal end taken 30 mm. below the hydranth, and from the same stems as Group A.

II (16.5–18° C.)
- Group C. 2-mm. stems with the distal end taken two mm. below the hydranth.
- Group D. 15-mm. stems taken just below the 2-mm. stems of Group C.

III (16.5–18° C.)
- Group E. 5-mm. stems of very small diameter, taken two mm. below the hydranth.
- Group F. 5-mm. stems of very large diameter, taken two mm. below the hydranth and from the same colony as Group E.

IV (16.5–18° C.)
- Group G. The distal ends of 10-mm. stems taken two mm. below the hydranth and ligated in the middle with nylon thread.
- Group H. The proximal ends of the stems in Group G.

V (19–20° C.)
- Group I. The same as Group G, but taken from a different colony.
- Group J. The proximal ends of the stems in Group I.

After all of the stems had regenerated, the period of regeneration of each stem was divided into two parts: (1) the length of time between the cutting of the stem and the appearance of the thickening which is the primordium of the regenerating hydranth (= movement time), and (2) the length of time between the appearance of the thickening and the appearance of the constriction which marks the base of the regenerated hydranth (= differentiation time). (For drawings of the stages see Steinberg, 1954.) Since the object of the experiment is to distinguish between the time required for movement and the time required for differentiation, the onset of the "thickened" stage was chosen as the division between the two periods because (1) the only known process preceding it (except for the breakdown, near the two cut ends, of the endodermal ridges which traverse the length of the stem) is the tissue movement, and (2) Davidson and Berrill (1948) have shown that this is the stage at which one finds the first evidence of determination by the test of isolation of parts of the thickening, followed by self-differentiation. It should be mentioned that determination here is only with respect to the replacement of missing proximal structures. Missing distal structures are always replaced in *Tubularia*, even by the adult hydranth.

Experimental Results

1. The basis of the axial gradient and differences in rate of regeneration

The results of the experiments are summarized in Table I. The experiment (II) in which short and long pieces are compared is not satisfactory because 14 of the 20 two-mm. stems which comprised Group C regenerated as partial bipolar forms. In these forms, common in short pieces, the entire tissue of the piece transforms into a pair of hydranths fused at their bases. Such bipolars lack proximal tentacles and in some cases gonophores as well. It is impossible to get regeneration rates in such stems because the constriction which marks the base of a regenerated hydranth and the formation of which is used as the end point of regeneration never appears. As Table I shows, no significant differences were

found, either in the movement or the differentiation phase, between groups C and D, probably because of the small number of cases in Group C. However, in every other pair of experiments there is a large difference in movement time between the two groups of the pair, the group regenerating more slowly always being the one in which the tissue movement occupies more time. This is not the case with respect to differentiation, however. In experiments III, IV and V the differentiation time of both groups of the pair is the same. Experiment I presents a different picture. Group B differentiated *more rapidly* than Group A. This is indeed remarkable, since B is the more slowly regenerating group of the pair,

TABLE I

Mean time required, with the standard error of the mean, for the entire regeneration process and for its two component parts, tissue movement and differentiation, in the various paired experimental groups; p indicates the probability that the difference between the two groups of a pair is due to chance alone. Standard errors and probabilities were calculated by the use of Student's "t" test

Experiment	Group	Description	No. of stems	Hours for regeneration	Hours for movement	p	Hours for differentiation	p
I	A	Distal piece	19	22.6±0.3	14.1±0.2	$\ll 0.001$	8.5±0.2	$\ll 0.001$
	B	Proximal piece	19	27.9±0.4	21.6±0.2		6.3±0.3	
II	C	Short piece	6	68.3±7.9	32.0±4.4	$\simeq 0.1$	36.3±6.5	$\simeq 0.4$
	D	Long piece	16	53.8±3.7	24.2±2.1		29.6±3.0	
III	E	Narrow piece	16	48.5±4.6	22.5±2.2	$\simeq 0.005$	26.0±4.0	$\simeq 0.4$
	F	Wide piece	16	59.1±3.1	32.7±2.1		26.4±2.2	
IV	G	Distal end of piece ligated in middle	17	33.3±1.0	21.4±0.7	$\ll 0.001$	11.9±0.7	$\simeq 0.1$
	H	Proximal end of stems in Group G	18	46.5±1.0	33.0±0.8		13.5±0.7	
V	I	See G	19	41.2±2.9	25.8±2.8	<0.001	15.4±0.8	>0.9
	J	See H	15	75.2±5.3	59.9±5.0		15.3±1.7	

being composed of pieces taken from the proximal region of long, unbranched stems, while the pieces of Group A are taken from the distal region. Thus in the case of distal versus proximal pieces of long stems, the slower rate of regeneration of the proximal pieces is entirely due to a slower rate of distad movement of the tissue, preceding the establishment of the hydranth primordium. This means that the axial gradient, as manifested by graded differences in rate of regeneration, is in reality a gradient in rate of tissue movement rather than a gradient in general "metabolic rate" prevailing during the entire period of regeneration. That the physiological processes occurring during the differentiation of a new hydranth

are certainly not slower in the more proximal regions is clearly shown by the fact that in these experiments the differentiation process itself is actually more rapid in proximal than in distal regions. In the other experiments, also, dealing with two groups regenerating at different rates, the difference in rate of regeneration between the two groups is in every case due to a difference in the time occupied by the tissue movement. In no case is it due to a difference in the rate of differentiation.

2. Reverse movement, rate of differentiation, and dedifferentiation

Table I shows that groups taken from different colonies may regenerate at markedly different rates. (Each experiment was performed with stems from a separate colony from which both groups within the experiment were taken.) Individuals *within* a colony may also regenerate at widely different rates. Table II illustrates the amount of variation in regeneration rate which may exist between comparable pieces from stems taken from a single colony. This particular sample

TABLE II

An example, drawn from the protocols of Group F, to show the amount of variation in rate of regeneration which may exist among comparable stems from the same colony. The figures indicate the number of stems in each stage at the times given

Time in hours	Nothing	Optical density gradient	Distal thickening	Proximal ridges	Distal ridges	Constricted or emerged
16.5	15	5	0	0	0	0
21.0	12	4	4	0	0	0
25.5	11	4	3	2	0	0
36.0	2	1	12	3	2	0
40.0	1	0	9	7	1	2
47.0	1	0	4	8	5	2
55.0	1	0	3	4	4	8
71.0	0	0	0	2	1	17

(Group F) was selected because it possessed the greatest amount of variation of any of the groups in these experiments. Not only does rate of regeneration considered as a whole vary, but the rate of differentiation also varies widely from colony to colony, as shown in Table I, and between different individuals from the same colony, as shown by the fairly sizeable standard errors for differentiation in some groups.

The protocols of the experiments show that neither the tissue movement nor the differentiation is always continuously progressive. In a large number of cases the tissue movement has been found to stop and then to reverse itself. In fact, regeneration at the proximal end of a piece ligated in the middle or of a long piece in which dominance does not act is dependent upon such a reversal in the direction of movement of the tissue (Steinberg, 1954). This is usually true in *T. crocea,* with which the present experiments were performed. However it is invariably true in the species (probably never identified) used for the experiments just referred to, because in that species the tissue movement is much more pronounced than it is in *T. crocea.* In yet a third species (referred to by Mr. Gray

of the Supply Department of the Marine Biological Laboratory as "the long, white kind") the movement is equally pronounced, while in a fourth species (referred to as "the short, brick-red kind," and probably *T. tenella*) the movement does not occur—a fact which has marked effects upon the phenomenon of dominance. In addition to reverses in the direction of movement, many cases have also been observed in which the differentiation process came to a standstill and then reversed. In the most extreme case a stem in the distal tentacle ridge stage, on the verge of constricting, began to dedifferentiate and continued this process until all traces of differentiation disappeared, including the thickening which had marked the primordium of the regenerating hydranth. At the same time it was observed that the tissue was moving back toward the proximal end of the piece. Much later a new primordium appeared and this time regeneration was completed. Dedifferentiation in *Tubularia* has been described before (Driesch, 1897, 1908; Peebles, 1900), but only as one possible aftermath to the cutting of the differentiating primordium. In the present cases it appears as a not infrequent occurrence in normal regeneration.

The observation that during the dedifferentiation of one primordium the tissue was undergoing a shift back toward the proximal end suggested that the many cases of dedifferentiation might be attributable to such movements. This seemed reasonable because (1) differentiation does not begin until a certain minimal concentration of cells has been accumulated at the presumptive regeneration site, and (2) the degree of regulation within a one- or two-mm. piece is also dependent upon cell number (Steinberg, 1954). This suggests that the continued differentiation of the primordium might be very sensitive to a decrease in its cell density, such as would occur if the tissue of the stem were to move away from it during the course of its differentiation. A direct test of this hypothesis by observation is not always possible because only rather gross movements of the tissue can be detected. However a rough test can be made by plotting the mean time for differentiation in a group against the number of reverses per stem in the movement phase. There are difficulties in the preparation of such a graph. It is not easy to decide in what cases a reversal in the direction of movement as noted in the protocols is truly a reversal and in what cases it merely reflects the fair amount of subjectivity involved in such observations. As has been mentioned before, in order to preserve as much objectivity as possible, the previous recordings for a regenerating stem were never looked at when a new recording was being made. As a result a considerable number of borderline cases are placed on one side of the line in one recording and on the other side in the next. Quite obviously these cases cannot be considered as true reverses in the movement of the tissue. Therefore the following three alternative criteria were established for the validity of reverse recordings. (1) Both the original stage and the subsequent reverse in the movement must be established by at least two consecutive similar recordings for each stage; (2) there must be at least two consecutive recordings which progress in the reverse direction; or (3) the recorded reverse in the movement must be of such magnitude that it could not be attributed to a difference in judgment. When these criteria are rigidly applied, the number of reverses is reduced so drastically that the maximum number in any group is four (Group D), with other groups yielding two reverses (Groups C, F and J), others yielding only one (Groups E, H and I), and the remaining groups yielding none (Groups A, B and G). These

criteria, although they are the best that could be devised, are for two reasons not nearly adequate for the establishment of an accurate correlation between reverse movements and the mean rate of differentiation. (1) In some groups there is a very large number of reverse recordings which do not meet any of the above criteria for significance, while in other groups there are none or very few. This

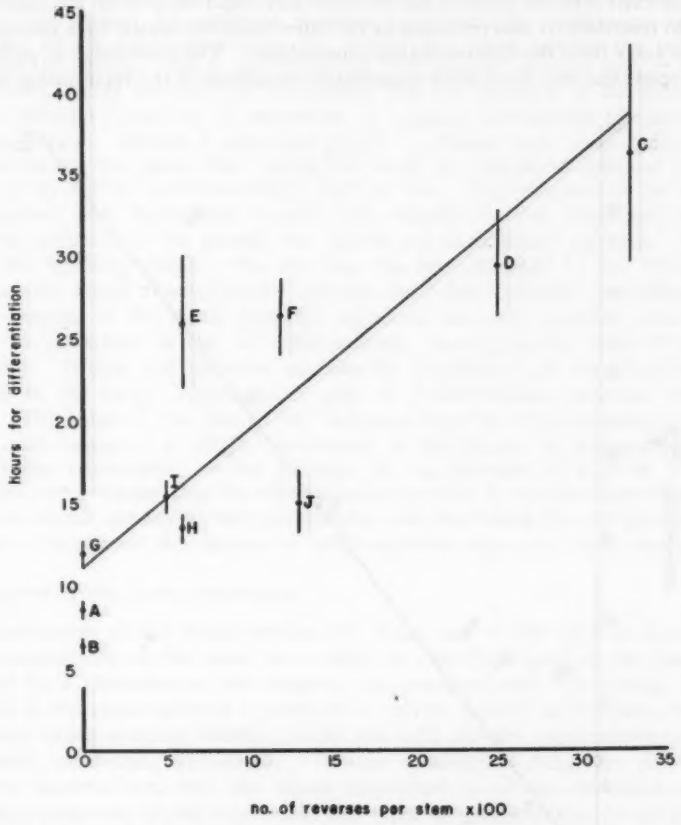

FIGURE 1. Correlation between the number of reverses during the movement phase and the time required for differentiation in the various groups. The line was arrived at by the Method of Least Squares.

can only mean that many reverse movements of short duration occur in some groups but cannot be distinguished from reverse recordings which are attributable to differences in judgment. (2) The number of reverses that meet one of the three criteria for significance is so small that it is bound to introduce large errors in the calculations. Nevertheless a very rough correlation between reverse move-

ments and rate of differentiation should be found if such movements really act to retard or reverse the differentiation process. That this rough correlation does indeed exist is shown in Figure 1. There is a general tendency for groups showing a higher percentage of reverses in the movement phase to show a slower mean rate of differentiation.

From the above we see that at least part of the differences in rate of differentiation which exist between colonies and between individual stems from the same colony are due to retardations and reversals in the differentiation caused by a movement of the tissue away from the differentiating primordium. The knowledge of such a correlation opens the way for a more quantitative treatment of the relationship between

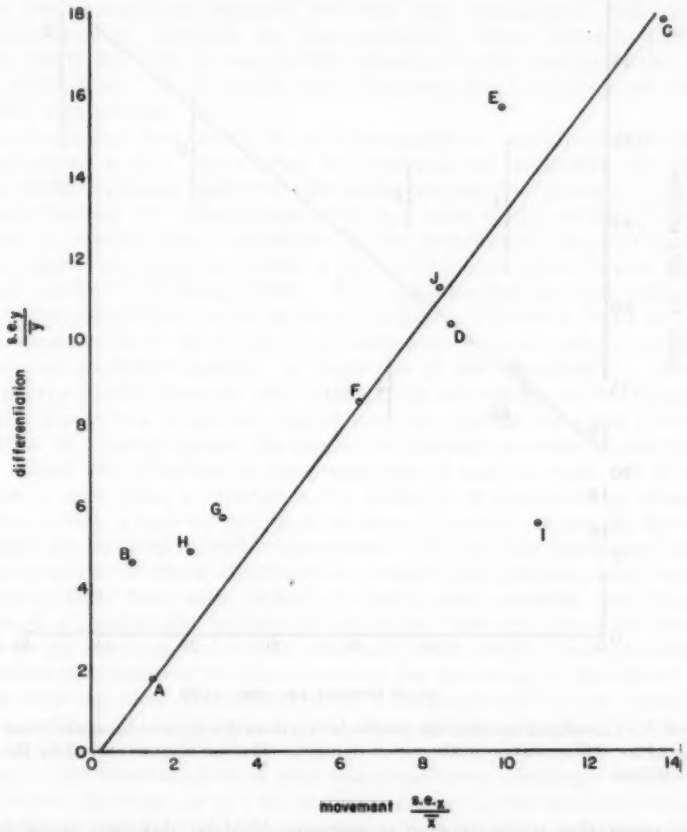

FIGURE 2. Correlation between the variation in time required for movement and the variation in time required for differentiation in the various groups. $\bar{x}$ = mean time for tissue movement; S.E.$_x$ = standard error of the mean for movement; $\bar{y}$ = mean time for differentiation; S.E.$_y$ = standard error of the mean for differentiation. The values on both axes are multiplied by 100. The line was arrived at by the Method of Least Squares.

variations in tissue movement and variations in rate of differentiation among similar stems from the *same* colony. The variation in tissue movement among the stems in a group can be numerically expressed by the figure $S.E._x/\bar{x}$, where $\bar{x}$ is the mean time for movement and $S.E._x$ is the standard error of the mean. Similarly the variation in rate of differentiation in a group can be expressed by $S.E._y/\bar{y}$, where $\bar{y}$ is the mean time for differentiation and $S.E._y$ is again the standard error of the mean. Since the variation in rate of differentiation has been shown to be caused, at least in part, by variations in tissue movement, the relationship between the two can be expressed by plotting the values of $S.E._x/\bar{x}$ against the values of $S.E._y/\bar{y}$ for the various groups and fitting the best possible straight line to the resulting points. This involves the assumption that the tendency of the stems in a group to undergo variations in movement is constant throughout the entire regeneration period. Should a particular group by chance show more variation in movement during one phase than during the other, the calculated value of $S.E._x/x$ for that group will be correspondingly high or low. Nevertheless, as far as can be determined, such fluctuations should occur equally in both directions, so that the over-all accuracy of the plotted line should not be seriously affected. Figure 2 shows the resulting graph. The fact that the line, obtained by the Method of Least Squares, passes almost directly through the origin indicates that differences in the movement of the tissue probably represent the *only* systemic factor contributing to variations in rate of differentiation among similar stems from the same colony. It does not, however, exclude the possibility that other factors may contribute to the large differences in rate of differentiation between *different* colonies. The slope of the line (1.35) indicates that the differentiating primordium is quite sensitive to slight movements of the tissue, an increase of 1 in $S.E._x/\bar{x}$ being responsible, on the average, for an increase of 1.35 in $S.E._y/\bar{y}$. This constitutes evidence that the differentiation process is continuously dependent upon a critical cell density in the primordium, and that when the cell density falls below this critical level the process of differentiation stops and even reverses.

3. The nature of the tissue movement

The movement of the tissue toward the distal end of the stem is due to an apparent contraction of the tissue as a whole in the distal part of the stem, accompanied by a stretching of the tissue in the proximal part (Steinberg, 1954). The result is the appearance of a gradient in optical density of the stem, the optical density being greatest distally, where the cells are the most numerous, and progressively decreasing proximally. It was considered likely, in view of a number of observations, that the tissue movement is of an amoeboid nature. Amoeboid movements of two ectodermal cell types of gymnoblasts, the cnidoblasts and the interstitial cells, have been described by Hadzi (1909), Kuchner (1934), Moore (1952), and Spratt (unpublished). The author has observed the amoeboid activity of the ectoderm of *Cordylophora, Eudendrium* and *Pennaria,* but has found no evidence of amoeboid movement of the endoderm. This situation seems to be reversed in *Hydra* (Papenfuss, 1934; Lehn, 1953), but seems to be rather general among the gymnoblasts.

In order to determine whether the distad movement of the tissue is of an amoeboid nature, the following experiment was performed. Twenty-five 7½-mm. stems were cut and all of the tissue was extruded from the perisarc with the ex-

ception of a band of tissue 1½ mm. long in the middle of the stem. If the movement of the tissue is of an amoeboid nature, the entire length of tissue should move toward the distal cut end of the perisarc. In 20 of the 25 stems the tissue did indeed move toward the distal cut end of the perisarc, traversing the three-mm. distance in the course of about 6 hours. In two stems the tissue moved in both directions, completely occupying the 7½-mm. length of perisarc in approximately the same length of time, while in only one case did the tissue fail completely to move. The tissue of the remaining two stems underwent incomplete distad movement. In many of the stems an optical density gradient appeared during the course of the movement. Two attempts were made to repeat this experiment, 5 stems being used each time, for the purpose of obtaining a photographic record of the movement, but each time the tissue moved toward both ends of the perisarc equally. The most likely explanation of this is that in both of the latter attempts colonies were used which had been in the laboratory for two or three days, such colonies giving aberrant results in many different types of experiments. An examination of the advancing edge of the tissue showed that it was composed of a very thin sheet of cells moving on the inner wall of the perisarc. At some points it could be seen that the extreme margin was a single layer of ectoderm cells, the endoderm terminating a few cells behind the advancing edge.

It is concluded that the movement of the tissue during the first phase of regeneration in *Tubularia* is an amoeboid movement of the ectoderm, the endoderm presumably being carried along passively because of its firm adhesion, by means of the mesoglea, to the ectoderm. Whether all of the cells of the ectoderm are amoeboid or whether the movement is due to the activity of certain specific cell types cannot be said at this time. It follows that the axial gradient in *Tubularia,* as manifested by graded differences in rate of regeneration, is a reflection of a gradient in the rate of movement of the amoeboid cells of the ectoderm.

Discussion

It is claimed by Tardent (1952, 1954) that the interstitial cells of hydroids are of great importance in the regeneration process and that the observed apico-basal gradient in the number of interstitial cells is the basis of the axial gradient in rate of regeneration. In the first paper it is stated that there is no longer any doubt that these cells migrate toward the cut surface where they form the "blastema." The author presents no observations to support his statement, relying upon claims to this effect in the literature. Perhaps the experiment most suggestive of such a phenomenon is that of Evlakhova (1946), in which the basal halves of normal individuals of *Hydra attenuata* were grafted to the apical halves of x-irradiated individuals of *Pelmatohydra oligactis* which are incapable of regeneration and tend to disintegrate. Regeneration followed amputation through the irradiated *Pelmatohydra,* the interstitial cells and cnidoblasts of the latter now being found to be those of *H. attenuata.* This, however, does not constitute evidence that the interstitial cells *formed* the regenerant. It is well known that the presence of non-irradiated tissue can vitiate the effects of x-irradiation elsewhere in the system, possibly by the contribution of -SH compounds (Barron, 1946; Jacobson *et al.,* 1949; Chapman *et al.,* 1949; Patt *et al.,* 1949; Abrams and Kaplan, 1951; Allen, 1951; Gershon-Cohen *et al.,* 1951). In his second paper Tardent acknowledges

(p. 628) that in *Tubularia* there is no such thing as a regeneration blastema and briefly considers the possibility that the other cells of the tissue may also contribute to the regenerant. However if one isolates a one-mm. length of stem, the entire piece transforms directly and *completely* into hydranth structures. This would appear to be a clear demonstration that the process of regeneration involves *all* of the cells of the regenerating region. The postulate that the axial gradient in rate of regeneration is due to a gradient of the cellular precursors of the regenerant is clearly inconsistent with the above observation.

The only existing theory up to the present time which attempts to explain the determination of the size of the hydranth primordium and the time required for regeneration is that of Barth (1944). This author points to the fact that both primordium size and the regeneration rate can be decreased by exposure to low oxygen tensions during the course of regeneration. He shows further that stems consume less oxygen at lower oxygen tensions and concludes that since energy production would be a function of oxygen consumption, both primordium size and regeneration rate are determined by the rate of energy production. In the light of the new facts concerning amoeboid movement of the tissue during regeneration and its importance in determining regeneration rate, a reinterpretation of Barth's data becomes possible.

In Barth's paper we read (p. 361), "During from 5 to 22 hours of exposure to N_2 sea water the stems appear to undergo no regeneration, since, when they are returned to O_2 sea water, they take the same time for regeneration as freshly cut stems." From this we can conclude that the amoeboid movement of the tissue cannot proceed anaerobically. Considering this, plus the fact that both primordium length and regeneration rate are determined during the movement phase of the regeneration process, it would appear that the action of low oxygen tension in reducing the primordium length and the regeneration rate is upon the amoeboid movement of the tissue, which would slow down and eventually stop as the oxygen tension is decreased. Barth also points to Peebles' finding (1931) that after 15 hours of regeneration the as yet unformed primordium could no longer be reduced in size by removing a large piece from the proximal end of the stem, that between 6 and 13 hours some of the primordia could be reduced in size, and that from two to four hours the size of all of the primordia could be reduced. He correlates this with his own finding that exposure to low oxygen tensions for various periods during the course of regeneration can decrease the size of the primordium, and concludes (p. 364) that "The results show that determination of the length of the primordium is progressive with time. There is no evidence of a sudden determination at some definite time. On the contrary, it is possible to change the length of the primordium during most of the period of regeneration. This implies that the distal parts of the hydranth are determined first and the more proximal parts later, for the changes in length of the primordium mean a change in the amount of stem derived from the proximal regions. Obviously, this proximal region forming the base of the hydranth must remain undetermined until the final few hours of regeneration." Since it has been found that the length of the primordium (and the rate of regeneration) is determined during the movement phase of regeneration and *some time before* the primordium is established, while determination of the parts of the regenerating hydranth cannot be detected until *after*

the primordium is established, it would seem more fruitful to consider these as two separate processes. If this is done, we can no longer consider that regional functional determination within the primordium proceeds basipetally or that the basal region of the hydranth remains undetermined until regeneration is nearly complete.

The statement that the length of the primordium can be changed during most of the period of regeneration is open to question and might best be discussed in connection with another experiment in Barth's paper. Stems were cut and divided into four groups. The first group, which served as a control, was allowed to regenerate under normal conditions. The second, third and fourth groups were treated with 0.07 *M* urethane for the periods from 0–10, 10–20, and 20–30 hours after cutting, respectively. The results show that regeneration in these groups was delayed 13.7 hours, 17.4 hours, and 4.4 hours, respectively, while the primordium length was also decreased, although not in proportion to the delay in regeneration. This latter fact is used as evidence that primordium length and regeneration rate are controlled by factors operating at different times. This conclusion is at variance with other findings already presented, but it might be pointed out in addition that when stems regenerate at low temperatures, regeneration rate is decreased (Moore, 1910; Moog, 1941) while primordium length is increased (Moog, 1941). It may be that at low temperatures the initiation of differentiation is delayed more than the tissue movement, so that more cells have a chance to enter the region in which reconstitution will occur before the differentiation begins, resulting in an increase in the size of the primordium. A similar effect may explain the lack of parallelism between regeneration rate and primordium length in Barth's urethane experiment.

An explanation is needed for the fact that Barth has reported an effect of low oxygen tension and of urethane on the length of the primordium well beyond the time at which both Peebles and the author have found the length to be no longer alterable by the removal of a large proximal piece of the stem. The following discussion may serve to bring these apparently contradictory findings into closer agreement. In Barth's experiments the stems were treated with urethane during specific intervals of time rather than during specific stages in their regeneration. Table II shows the wide spectrum of stages in which similar stems from the same colony may be at any given time. Admittedly the variation shown in Table II is the largest found in any of the groups in the present experiments, but the fact remains that there may be a considerable spread in regeneration rate among the stems of an experimental series. Treatment during specific intervals of time, then, may result in different effects upon different stems, depending upon their stage. This would not be revealed in Barth's data because he presents his results as averages. If there were a very specific stage at which the treatment is effective, the effect would be manifested as one which gradually appears and then gradually disappears, due to the use of averaged data and specific time intervals rather than specific stages. This is actually shown by the results. Finally, the time intervals during which the animals were treated are referred back to the time period for normal regeneration and used as a measure of the sensitivity of the controls to urethane treatment. However, if stems treated for the period between 10 and 20 hours after cutting are delayed an average of 17.4 hours, the treatment has not only stopped all regeneration at around 10 hours, but has even occasioned

the necessity of a mean recovery period of about 7.4 hours. It can be inferred from these results that the control stems were highly susceptible to the treatment at 10 hours, but no inference can be drawn concerning their susceptibility at, say, 15 or 20 hours because the treated stems did not pass through a comparable stage until some time after the cessation of the treatment. It would appear, then, that the effects of oxygen and of urethane upon regeneration rate and primordium length may be interpreted as having terminated at an earlier time than would at first be suspected. "Determination" of the length of the primordium would be expected to proceed gradually after the tissue movement has begun, and to terminate at the time when a certain critical concentration of cells has been reached at the presumptive regeneration site. It may be stated in summary that all existing evidence is consistent with the view that both the regeneration rate and the primordium length are determined during the phase of tissue movement and before the appearance of the primordium. One qualification should be added, however. We have seen that a reverse movement during the course of differentiation may slow down, stop, or even reverse the differentiation process. This being the case, the determination that we speak of is at best a labile one, and in the very strictest sense it would not be true to say that the regeneration rate of a particular stem is fixed when the primordium makes its appearance. This does not, of course, alter the fact that differences in regeneration rate between two *groups* of stems from the same colony, such as have been studied in the present experiments, are entirely due to differences in the time occupied by the movement (pre-differentiation) phase of the regeneration process.

There appears to be good evidence that there is a gradient in oxygen consumption, high distally and decreasing proximally, in the tubularian stem. Hyman (1926), Barth (1940), and Sze (1953), using a modified Winkler method, a Warburg respirometer, and the Cartesian diver method, respectively, have all found this to be true. Certain objections can be raised to these experiments, such as the number of approximations made by Hyman, the inclusion by Barth and Sze of the perisarc, which is thicker proximally, when taking dry weights, and the unusually small number of cases (14 stems divided among three experiments) in the experiments of Sze. Nevertheless, even allowing for these objections, it still appears that a respiratory gradient exists. If respiratory rate can be assumed to be a good measure of the physiological processes occurring, what explanation can be offered for the lack of an apico-basal gradient in rate of differentiation? In the present experiments a gradient in rate of differentiation has been found, but it goes in precisely the opposite direction. A possible explanation is to be found in the measurements made by Barth (1940) on oxygen consumption during the course of regeneration. This author finds (p. 369) that "The (respiratory) rate is highest from about 7 to 16 hours and it is during this period that the size of the primordium is determined (Peebles, '31). These changes in rate are observed only in the case of short (2–4 mm.) stems. The S-shape of the curve is lost or almost lost when the data from long (8–15 mm.) stems are plotted." It has been found (Steinberg, 1954, and the protocols of the present experiments) that in short stems the tissue moves toward the distal end and then ceases movement, and that after a hydranth regenerates distally it produces an inhibitor which prevents further movement. In long stems the tissue continues to move, even after the regeneration of a hydranth distally, presumably because of the dilution

of the inhibitor below an effective concentration by the increased volume of the coelenteric fluid. These observations correlate perfectly with the observations of Barth quoted above if one assumes that the differences in oxygen consumption which appear are measurements of the differences in the movement of the tissue. It has already been inferred that the tissue movement is a process which requires oxygen. Thus we see that oxygen consumption is higher distally than proximally, and the tissue moves more rapidly distally than proximally; oxygen consumption is highest in short stems from about 7 to 16 hours after cutting, and the tissue movement is occurring in short stems from the average colony at this very time; the above peak in oxygen consumption is not found in long stems, and the tissue does not stop moving in long stems. It would appear, then, that the gradient in oxygen consumption is in reality a measure of the rate of tissue movement in the different levels of the stem. However the question of what causes the cells from basal regions to move more slowly than those from apical regions must still remain a subject for future investigation.

Summary

1. The regeneration process in *Tubularia* is divisible into two phases. There is a period of tissue movement, during which extra cells are provided to the presumptive regeneration site, followed directly by the period of differentiation of the new hydranth.

2. Differences in rate of regeneration between narrow versus wide stems, the distal versus the proximal ends of stems ligated in the middle, and apical versus basal stem levels are all due to differences in the time occupied by the tissue movement.

3. Narrow and wide stems differentiate at the same rate, as do the distal and proximal ends of stems ligated in the middle. However, pieces from *basal* stem levels differentiate, in these experiments, at a significantly *greater* rate than pieces from *apical* stem levels.

4. Both rate of regeneration considered as a whole and rate of differentiation vary from colony to colony and between comparable stems taken from the same colony.

5. Neither the tissue movement nor the process of differentiation is always continuously progressive. Reverses in the direction of movement and spontaneous dedifferentiation of the differentiating primordium have both been observed.

6. The differentiation process is continuously dependent upon a critical cell density in the primordium. Evidence is presented that differences between stems in the movement of the tissue during the differentiation process represent the only systemic factor responsible for variations in differentiation rate among similar stems from the same colony.

7. The tissue movement is due to the movement of the amoeboid cells of the ectoderm, and the axial gradient, as manifested by an apico-basal decrease in rate of regeneration, is a reflection of the apico-basal gradient in the rate of tissue movement.

8. Both the apico-basal gradient in oxygen consumption and differences in oxygen consumption during regeneration correlate perfectly with the rates of tissue movement at different levels of the stem and with the period during which

the movement occurs. It is inferred that differences in oxygen consumption indirectly measure the differences in tissue movement, which is an aerobic process.

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INDUCTION OF MOLTING IN THE CRAYFISH, CAMBARUS, BY MODIFICATION OF DAILY PHOTOPERIOD[1]

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Several investigators have been concerned with the modifications in reproductive phenomena in the Crustacea which are induced by subjecting the animals to various daily light rations. Thus Stephens (1952) studied the modifications in oöcyte maturation in the crayfish, *Cambarus,* induced by exposure to various daily photoperiods and postulated a definite role of light in the control of normal reproductive cycles in these animals. Paris and Jenner (1952) extended the observation of photoperiodism in the Crustacea to the fresh water prawn, *Palaemonetes,* and also demonstrated that administration of the daily light ration in a discontinuous fashion may permit stimulation by daily rations which are not effective when administered continuously. Stephens (1953) provided evidence for an influence of day-length on the rate of development of secondary sex characteristics of the crayfish, *Cambarus.*

Hormonal influences on the crustacean sex cycle were first demonstrated by Panouse (1943, 1944, 1946) for the shrimp, *Leander,* and the accelerating effect of eyestalk removal in ovarian development was subsequently confirmed for *Cambarus* and the fiddler crab, *Uca,* by Brown and Jones (1947, 1948). Carlisle (1953) has recently demonstrated an inhibitory factor controlling sex reversal in *Lysmata.* The presence of a molt-inhibiting principle in the sinus gland-X-organ complex has been verified in a number of laboratories (reviewed in Brown, 1952) although this principle may not always be present (Carlisle, 1954) and Echalier (1954) provides evidence suggesting the occurrence of a molt-accelerating principle in the Y-organ of *Carcinus.*

Finally, Scudamore (1948) presents evidence for a functional interrelation of the processes of egg-laying and molt. In view of the hormonal influences on the molting process, the intimate relation between the molting cycle and the reproductive cycle, and the influence of light as an exteroceptive factor controlling reproduction, the possibility of the modification of molting by treatment with various light periods is of considerable interest.

The results of experiments involving exposure of *Cambarus* to various daily periods of illumination which had been undertaken for other purposes suggested that molt frequency might be influenced by this treatment. However, the numbers of animals involved were small and the results inconclusive so that it was thought desirable to conduct observations on a larger group in order to obtain a more reliable indication of a possible influence on molting.

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Material and Methods

The animals employed in the experiment to be described were *Cambarus virilis* collected in an artificial mud-bottom lake north of Chicago, Illinois. They were obtained in mid-September and maintained in the laboratory until early November in a large tank supplied with running water. During this time the tank was shielded with black cloth so that all of the animals were maintained in constant darkness. The animals were rather small (average carapace length about 2 cm.) and were probably immature yearlings. The oöcytes of the females were small and showed no sign of yolk deposition in contrast to the normal situation in mature *C. virilis* females at this season. Also the gonopods of the males were uniformly juvenile in form (form II) while those of mature males are typically form I (sexually active) at this time (Scudamore, 1948).

Early in November, these animals were divided into three groups and maintained under the following lighting conditions:

Group 1 consisted of one hundred and thirty animals which were placed in a tank shielded with black cloth to provide constant darkness.

Group 2 consisted of one hundred and ten animals subjected to normal day-length for the local latitude and season. This day-length varied from an initial maximum of ten hours and thirteen minutes through a minimum of nine hours and seven minutes on December 21st (data from the American Ephemerix and Nautical Almanac).

Group 3 consisted of sixty-five animals receiving twenty hours of light per day from 2 A.M. to 10 P.M. Lighting was provided by two 25-watt, unfrosted incandescent bulbs suspended from a wooden frame approximately ten inches above the water level. This produced an illumination of from 8 to 50 foot candles at the water surface. All tanks were covered with black cloth to prevent the access of undesired illumination and the lights were controlled by automatic time clocks.

The temperature in each tank was taken daily and the water flow was adjusted so that the temperatures in the three tanks were equal. Each tank was examined daily for dead animals and cast exoskeletons. As animals died they were autopsied and the gastroliths, if any, removed and weighed after drying. For some days preceding molt in crayfishes calcium is stored in the form of gastroliths in the anterior wall of the stomach. Scudamore (1947) has studied this cycle in animals induced to molt by removal of the eyestalks and demonstrates an increase in gastrolith weight as these animals approach the time of molt. Consequently gastroliths were weighed to provide some information concerning the position of the animals in the molt cycle. In order to compensate for variation in size of the animals a gastrolith factor was calculated and the results are recorded in this form in Table I. This gastrolith factor consists of the weight of the gastroliths in milligrams divided by the cube of the carapace length in centimeters. This quotient is multiplied by 100 for convenience.

Results

The animals in the three groups showed clear differences with respect to molt and gastrolith formation, and mortality.

Group 1, which was composed of thirty-seven females and eighty-three males, was maintained in constant darkness. This group showed the lowest mortality

and the least tendency toward gastrolith formation. The average temperature in this tank for the experimental period was 15.2° C. Seventeen animals died during the sixty-four-day experimental period which is a mortality of 13%. Only one animal of the seventeen which died showed any trace of gastrolith formation. This was a male which died sixty-three days after the beginning of the experiment and had a gastrolith factor of 24. No molts occurred in this group.

TABLE I

Record of the time of death or molt and the gastrolith factors for animals of group 2 (normal daylength) and group 3 (20-hour light)

Day	Group 2 (normal daylength)		Group 3 (20-hour light)	
	Sex	Gastrolith factor	Sex	Gastrolith factor
4	female	0		
6			male	0
8			male	0
9			male	0
			female	63
11			male	0
13	male	0	female	0
	male	0		
15	male	5	male	0
			female	0
16			male	0
			male	49
17	female	0	female	0
	male	0	female	trace*
	male	0	male	3
19	female	0	female	0
	male	0	male	0
	male	0		
20			female	131
			female	52
21			female	8
			female	18
23	male	0	male	0
	male	0		
	male	1		
24	male	2	female	0
27	male	0	male	0
	male	trace*		
	male	10		
	female	6		

* Too small to be isolated and weighed.

TABLE I—*Continued*

Day	Group 2 (normal daylength)		Group 3 (20-hour light)	
	Sex	Gastrolith factor	Sex	Gastrolith factor
29	male	0	male	0
			male	25
30			female	0
			male	trace*
32	male	0	male	0
			male	32
34	male	0	female	0
	male	14		
35			female	0
			female	56
38	male	6		
	male	21		
42	male	trace*	male	6
			male	35
44	male	successful molt		
	male	0		
	female	0		
	female	22		
48			male	successful molt
50			female	successful molt
			male	3
53	male	0		
55			female	0
59			male	successful molt
			male	(post-molt)
			male	38
			male	14
			female	0
61	male	15	male	successful molt
			female	successful molt
			male	1
64	male	0	male	successful molt

Group 2, a group of thirty females and eighty males, was exposed to normal day-length under the conditions described. The average temperature in this tank during the period of observations was 15.2° C. Thirty-one of these animals died during the experimental period which is a mortality of 28%. Twelve of the animals which died showed some development of gastroliths and one male molted forty-four days after the beginning of the experiment. Thus 40.6% (thirteen of thirty-two) of the animals examined showed some indication of molt or its successful completion. The average gastrolith factor for those animals which ex-

hibited gastroliths at the time of death, sex, and gastrolith factor or record of molt for the animals in this group are recorded in Table I.

Group 3, composed of twenty-five females and forty males, was exposed to twenty hours of light per day from 2 A.M. to 10 P.M. The average temperature in this tank was 15.3° C. Forty of these animals died during the observation period which is a mortality of 62%. Eighteen of the animals which died showed some sign of gastrolith formation and one of them died after having successfully molted. Six animals molted successfully and survived during the course of these observations. Hence 54.4% (twenty-five of forty-six) of the animals observed molted or showed an indication of the initiation of molt. The average gastrolith factor for those animals which exhibited gastroliths at the time of death was 29.5. The time of death, sex, and gastrolith factor or molt record for the animals in this group are listed in Table I.

The difference in behavior between the animals maintained in constant darkness (group 1) and groups 2 and 3 appears significant on inspection. This is verified statistically when one considers the percentage of the animals observed which show an indication of molt in the three groups. The difference between group 1 and groups 2 and 3 is more than twice the standard error of the difference. However, this does not suffice to demonstrate the significance of the difference between group 2 and group 3 (difference is 1.2 sigma). Applying Student's t test after assigning a reasonable gastrolith factor to the successful molts, the probability that groups 2 and 3 are not different is less than 0.01. The factor value of 150 which was assigned to animals which molted is probably somewhat low; higher values would emphasize the difference between the two groups.

Concerning the differences in mortality of the three groups, all the differences are significant (greater than twice the standard error of the differences) provided one includes the animals which showed gastroliths at the time of death. However, after these animals are excluded, the death rate of the animals exposed to twenty hours of light per day is still significantly higher than that of the animals in groups 1 and 2.

Discussion

It can be concluded on the basis of these results that exposure of *Cambarus virilis* to rather low light intensities during the winter months is capable of inducing molt in animals which would fail to molt if maintained in constant darkness. It is interesting to note that apparently rather short day-lengths are competent to produce this effect. Light periods to which the group in normal day-length, group 2, was exposed varied from a maximum of ten hours and thirteen minutes to a minimum of nine hours and seven minutes. This is in contrast to the results obtained by Paris and Jenner (1952) where a light period of 11 hours per day failed to induce ovarian development in *Palaemonetes*. These authors conclude that the response of these animals is in many respects analogous to the flowering response in long-day plants. On the other hand, Stephens (1952) observed modifications of yolk deposition and oöcyte maturation induced by rather short day-lengths although the situation is somewhat complicated by the fact that her initial exposures were longer (twelve hours) and decreased gradually.

In the results presented here, it would seem that the difference between the response of the animals to short days and twenty-hour days is quantitative rather

than qualitative. If there is a threshold for this response, it lies below ten hours and thirteen minutes.

It does not seem likely that photoperiodic responses can provide a complete explanation for the timing of the events in the molt cycle but it does appear that they would contribute in this regard. The typical molt cycle in *Cambarus* consists of a variable number of molts in the first year followed by an annual molt of the females and a spring and fall molt of the males in succeeding years (van Deventer, 1937). The transition from darkness or very short daily exposures to light to day-lengths of twelve hours or more when the animals emerge from their burrows in the spring would provide a reasonable stimulus for the spring molt in both sexes. It is tempting to refer to the well-known retiring behavior of berried females as a possible factor in the delayed spring molt of this group which typically occurs two to three weeks later than the male spring molt. It would be possible to construct hypotheses to attempt to explain the rest of the molt cycle on the basis of variations in susceptibility to light as a stimulus or quiescent periods but there is not sufficient information available to limit the number of such schemes. Furthermore, the possibility that this response is characteristic only of immature animals cannot be discarded at present.

It should finally be pointed out that the results presented show a clear influence of day-length on the viability of these animals. There is a significant increase in the mortality of those animals which did not show gastroliths at the time of death as well as a clear increase in total mortality with increasing day-length. The fact that this increase occurs in animals which do not show gastroliths might suggest that the increase in mortality is not causally related to the increased tendency to molt. However, such a conclusion would rest on the statement that no animal which fails to exhibit gastroliths has entered the physiological pre-molt stage; this does not seem justified. However, it is of interest to compare this increase in mortality with the results of destalking reported by Brown (1938) which led him to postulate a hormonal effect on viability in crayfishes.

Summary

1. During the winter months, crayfishes maintained at moderate water temperatures (approximately 15° C.) respond to daily illumination by an increased tendency to molt.

2. The strength of this tendency to molt increases with increasing length of the daily periods of illumination.

3. If there is a threshold below which this response does not occur, it lies below a daily period of illumination of approximately ten and one quarter hours per day.

4. There is an increase in mortality in groups exposed to various daily photoperiods with the highest mortality occurring in animals exposed to the longest light period. This is true of animals which fail to show any sign of the initiation of molt at the time of death.

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DESOXYRIBOSE NUCLEIC ACID CONTENT IN THE NUCLEI OF SALAMANDER SOMATIC TISSUES

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Irregularities of ploidy in amphibians have been extensively investigated by Parmenter (1933, 1940), Fankhauser and his associates (*cf.* reviews by Fankhauser, 1945, 1952), and Costello and Henley (1949, 1950; Henley and Costello, 1951). These studies have dealt principally with conditions in early embryos and in the epithelium of larval tail-tips. Recently increasing attention has been given to the incidence of endopolyploidy in the adult somatic tissues of vertebrates, of which the mammalian liver and orbital glands have become classic examples (Sulkin, 1943; Biesele, 1944; Swift, 1950; Teir, 1944). The extent of such nuclear deviations in the normal tissues of adult amphibians has received only slight study. Mancini (1945) found a very rare occurrence of large nuclei in the liver of old salamanders. In the renal tubule cells of the Australian desert frog, *Cyclorana*, Dawson (1948) found striking variations in numbers and sizes of nuclei; a similar condition was reported by Schreiber and Melucci (1949) in the South American frog, *Leptodactylus*. In the integumental granular glands of *Triturus viridescens* Dawson (1937) described great volume changes of nuclei associated with secretory activity. Though changes in nuclear size often reflect corresponding changes in chromatin content, this relationship does not invariably hold. Chromosome counts are not feasible in adult tissues showing little or no mitotic activity. Cytospectrophotometry, however, affords a means for measuring the relative content of desoxyribose nucleic acid (DNA) in individual interphasic nuclei and thus provides a valuable technique for the determination of polyploidy and related conditions (*cf.* review by Swift, 1953). The present investigation was undertaken to provide such photometric data for adult tissues of the salamander *Taricha* (*Triturus*) *granulosa granulosa* Skilton. Parenchymal cells of the liver, pancreas, renal tubules, and granular glands of the skin were selected for study.

Methods

Pieces of liver, pancreas, kidney and skin from freshly collected water-inhabiting salamanders were fixed in 30 per cent neutral formalin. Paraffin sections were cut at thicknesses exceeding by two or three micra the average diameters of the constituent parenchymal nuclei. Sections of liver tissue from the same block were placed on all slides as a standard of photometric comparison. Staining with the Feulgen reagent followed the procedure described by Stowell (1945). The time

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of hydrolysis in 1 N HCl at 60° C. was 20 minutes, though a difference of five minutes did not produce an appreciable change in the maximal intensity of the color produced by the subsequent treatment with the sulphurous fuchsin reagent for one hour. Unhydrolyzed sections were also stained for controls. All the slides measured were treated with the same stock Feulgen reagent kept completely colorless by storage in tightly closed containers at 5° C.

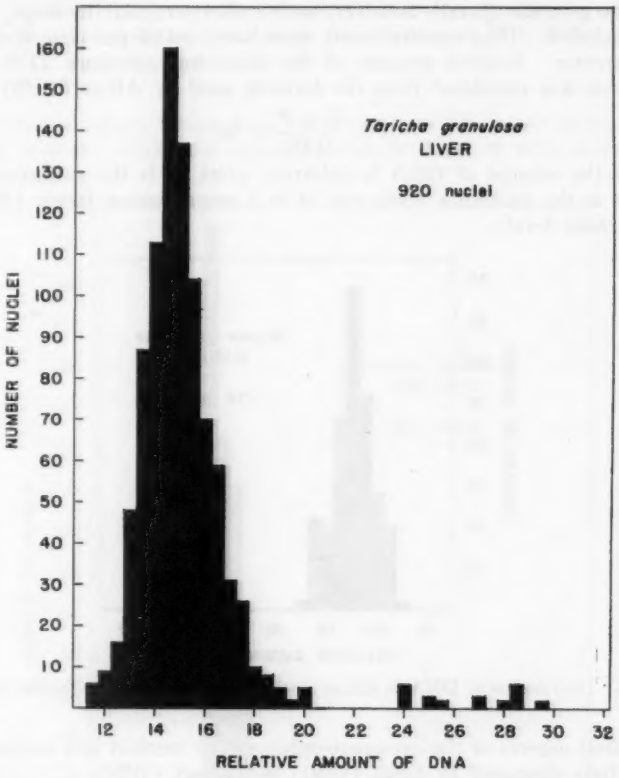

FIGURE 1. Distribution of DNA in liver nuclei of the salamander, *Taricha granulosa*.

Photometric measurements were made with an apparatus similar to that described by Swift (1950). Light of 550 mμ was isolated from a tungsten filament by a Bausch and Lomb interference filter. Optics on centerable mountings were carried on a Leitz microscope stand provided with a side-tube, and consisted of a B. & L. 4 mm. apochromatic objective (N.A. 0.95), a Leitz aplanatic, achromatic condenser (N.A. 1.40) stopped to 5 mm. diaphragm aperture, and 6 × or 8 × Leitz periplan oculars. Transmissions were recorded with a Farrand electron multiplier photometer, employing an RCA 1P21 phototube and a Rubicon galvanometer. The

phototube housing was mounted on a Leitz Aristophot camera. A calibrated iris diaphragm directly under the phototube permitted restriction of the field to any desired area. Measurements of nuclear diameters at predetermined magnifications were conveniently made on the reflex screen of the Aristophot camera.

Care was taken to measure only entire nuclei as determined by critical focusing. Since most nuclei in the present material were approximately spherical, the "plug method" for cytophotometry proved adequate and was used throughout; the largest nuclei of the granular glands, however, were rather irregular in shape, and hence were not included. Plug measurements were based on 60 per cent of the average nuclear diameter. Relative amount of the absorbing substance (DNA) in the whole nucleus was calculated from the formula used by Alfert (1950), *viz.:*

$$A = \frac{E \times r^2}{0.49} \times M^2$$

where A is the amount of DNA in arbitrary units, E is the extinction, r is the plug radius at the phototube level, and M is a magnification factor ($M = \mu$/mm. at the phototube level).

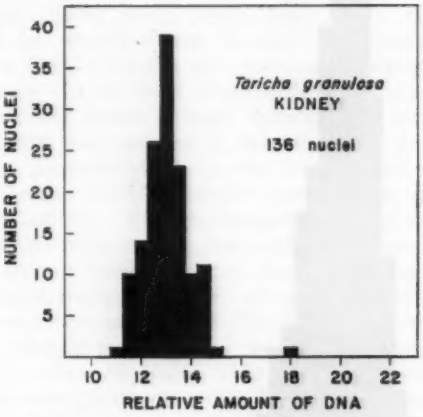

FIGURE 2. Distribution of DNA in kidney nuclei of the salamander, *Taricha granulosa*.

Theoretical aspects of the cytospectrophotometric method and sources of error have been fully discussed by Swift (1950) and Alfert (1950).

In order to obtain a reasonably representative number of determinations for the nuclei of the four tissues, all of the whole nuclei included in any one section were measured, except where deviations of shape did not allow their inclusion, as in the granular glands.

RESULTS

Liver

Interphasic nuclei of liver parenchyma were measured in two animals. The distribution of the relative amounts of DNA was similar in both, so that only one is illustrated (Fig. 1). In this specimen 920 nuclei were measured, in the other

644. Within the ranges 11.5 to 20 and 12 to 20, respectively, the great majority of DNA values formed in both cases a high, rather sharply delimited and fairly symmetrical unimodal curve. A few DNA values, 2.6 per cent of the total number of determinations in the animal figured and 2.2 per cent in the other, were scattered from 24 to 29.5 and from 22 to 33, respectively. The DNA values of liver nuclei were thus grouped into two classes. The peak value for class I nuclei was 14.5 in both specimens; respective means and standard deviations were 14.9 (1.4) and 14.8 (0.9). Class II nuclei, which included DNA values double the class I peak, showed means and standard deviations of 26.5 (2.0) and 25.3 (2.6).

Kidney

The number of kidney tubule nuclei measured was 136 in one animal (Fig. 2) and 129 in another. Unimodal curves similar to those of liver were obtained.

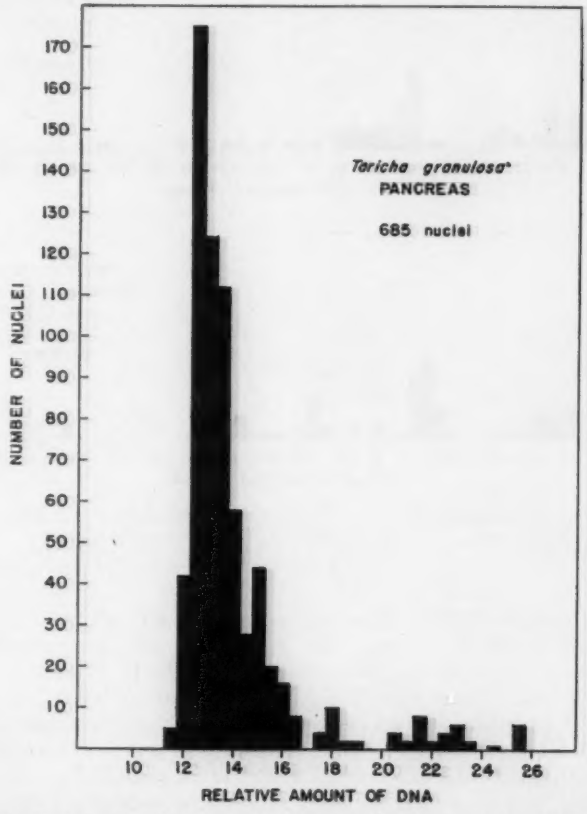

FIGURE 3. Distribution of DNA in pancreatic nuclei of the salamander, *Taricha granulosa*.

In both animals all DNA values ranging from 11 to 18 and from 11.5 to 19.5, respectively, with a peak value of 13.0 in both cases, could be placed into class I; means and standard deviations were 13.0 (0.9) and 13.3 (1.5). In the second specimen two DNA values fell at 21, but none twice the peak amount of class I were found. Careful scanning of several sections did not show any large and darkly stained nuclei which might give class II values. The average DNA content of 13.5 for 10 nuclei of the standard liver section which was mounted together with the kidney sections for comparison, seemed to indicate a slightly lower intensity of stain in the kidney slides. The comparison, however, also showed that class I nuclei of kidney cells were in close agreement with class I nuclei of liver cells with respect to DNA content.

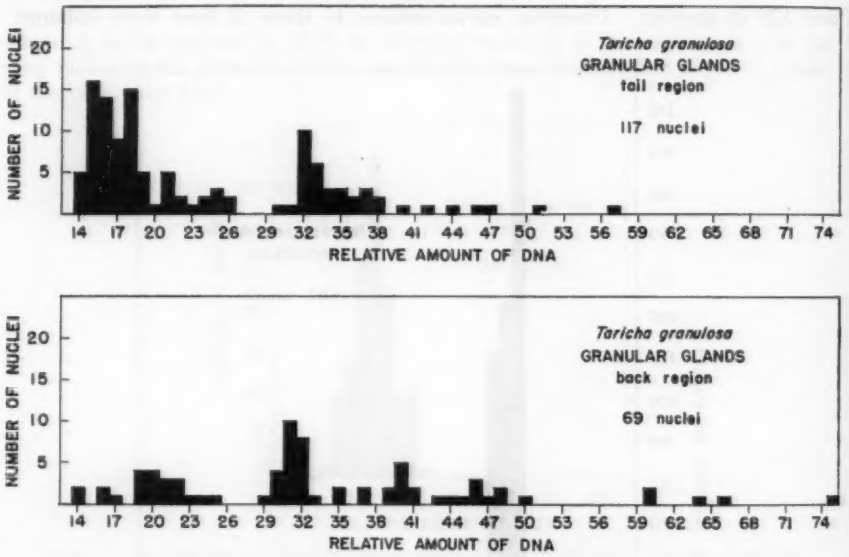

FIGURE 4. Distribution of DNA in the granular gland nuclei of the tail and back regions of the salamander, *Taricha granulosa*.

Pancreas

The distribution of DNA values was determined for 685 nuclei in one animal (Fig. 3) and for 944 in another. The results obtained were essentially similar to those found in liver nuclei. Within the ranges 11 to 19 and 11.5 to 19, respectively, the DNA values formed high unimodal curves (class I nuclei). Only a few values, 5.2 per cent and 1.6 per cent, respectively, of the totals were found in the ranges 20.5 to 25.5 and 20 to 24. The class I peak values occurred at 12.5 in the first animal (mean 13.5, st. dev. 1.3) and at 13.5 (mean 13.7, st. dev. 1.3) in the second. DNA content in the standard liver nuclei averaged 14.7 and 15.0, respectively. The slightly lower mean values of pancreatic nuclei can be accounted

for by their less homogeneous chromatin distribution. Class II means and standard deviations were, respectively, 22.6 (1.6) and 22.9 (1.3).

Granular glands

Skin sections were taken from the dorsal tail region in one animal and from the back region in another. In the first case most of the granular glands were at earlier developmental stages than in the second. In the younger glands the cell membranes were well defined, the cytoplasm was evenly filled with secretion granules, small nuclei were common, and most of the larger nuclei occupied a central position in the cell. A larger number of mature glands were found in the second location. In these the cell boundaries were lost (apocrine secretion), the cytoplasm was vacuolated, and peripherally located giant nuclei were numerous. These very large nuclei were usually flattened and could not be measured with the present photometric method. Because of this limitation only a small number of measurements were possible, 117 in the first animal and 69 in the second. Therefore the results, graphically shown in Figure 4, include only a partial representation of the granular gland nuclei.

A wide spectrum of DNA values was obtained in both specimens. In the tail region, where the smaller nuclei were most numerous, low DNA values were in the majority. In the back region, larger nuclei with high DNA values were relatively more abundant. Delimitation into nuclear classes is difficult. A possible grouping would include in class I the values 14 to 26, in class II 29 to 51, and in class III 57 to 75. Such a break-down would fit both graphs and would roughly yield successive doubling of mean values (I = 17.7 and 19.7; II = 35.5 and 36.5; III = 57 and 65). However, in comparison with the control liver sections, which showed means of 14.3 and 14.2, respectively, the above class I means are rather high. In part, this may be accounted for by the very homogeneous distribution of chromatin in granular gland nuclei, resulting in higher extinction values. If class I is taken to include only values 14 to 18, the means fall around 16 (in better agreement with the liver values), with doubling at 32 (at which peak groups occur in both graphs) and quadrupling at 64 (in harmony with the highest plots). This would leave considerable numbers of nuclei with intermediate values, notably the groups between 20 and 26 and between 39 and 51. Further consideration of this situation is deferred to the discussion.

Discussion

The nuclear DNA classes indicated by the distribution curves suggest a progressive doubling of chromatin. Class II nuclei might be interpreted as interphasic doubling prior to mitotic separation of diploid complements were it not for the fact that no mitoses were observed in the tissues examined. Hence it is more likely that these nuclei are definitively tetraploid or in an equivalent polytene condition. This conclusion gains strength in the case of the granular glands where class III (octoploid) values occur. The general picture is in agreement with similar phenomena in mammalian and other tissues (see Swift, 1953).

Variation within classes may be due in part to over-all error in the photometric technique, the exact value of which cannot be assessed but probably does

not exceed 15 per cent (Swift and Kleinfeld, 1953). Some of it might be accounted for by incomplete synthesis of DNA during endomitotic activity. The possibility of aneuploidy also cannot be disregarded, since its occurrence in the somatic tissues of mammals has been reported by Therman and Timonen (1951) and by Hsu and Pomerat (1953); Walker and Boothroyd (1954), however, believe that its incidence has been overestimated. In the absence of actual chromosome counts, the presence of aneuploid nuclei cannot be established.

The large number of individual nuclei measured in the several tissues of the present study permits estimation of polyploid frequencies. Unlike certain mammalian tissues, where polyploidy has been found to be common in beef liver and may even reach 50 per cent in old mouse liver, as reported by Swift (1950), salamander hepatic and pancreatic nuclei show very low polyploid frequency. Only about 2.5 per cent tetraploidy (class II nuclei) was found in the liver, 5.2 per cent in the pancreas, and no octoploid amounts in either. Kidney nuclei were entirely of the diploid class. The polyploid frequency, however, might vary with age of the animal, as is known to happen in mammals. Seasonal variation might also occur.

The granular glands of the integument present a case of special interest. The conspicuous nuclear changes in size, position and shape accompanying the various phases of secretory activity have been fully described by Dawson (1937) for the salamander *Triturus viridescens*. He thought, though the evidence was not conclusive, that these changes might suggest nuclear participation in the glandular activity. In the mature glands he noted nuclei of gigantic size, alveolar patterns of chromatin distribution, vacuolizations, and indications of possible passage of nuclear material into the cytoplasm.

In the present study, since a great part of the nuclear population could not be measured because of various shape irregularities, the account of the DNA distribution is not complete. There is, however, sufficient evidence that high polyploid (and possibly aneuploid) frequency exists, and an indication that it increases in degree as well as frequency during the development and maturation of the glands. This is exemplified by the greater prevalence of high DNA values in the older glands of the back region. Though only a few determinations were technically possible for nuclei in the octoploid range, large darkly staining and irregular or flattened nuclei, which might give similar or higher values, were frequently encountered. Thus it was not possible to establish the full extent of polyploidy in the mature glands. Giant nuclei which stained lightly were also observed, but their irregular shapes likewise precluded photometric determination by the present method.

In view of the considerable number of nuclei with apparent intermediate DNA values, it may be that chromatin multiplication is not wholly euploid. As already pointed out, mitoses are absent, so that direct evidence through chromosome counts could not be obtained. If polyteny is involved, it is conceivable that chromonemal duplication may be uneven. Or it may simply be that the intermediate values reflect a high rate of endomitotic activity, resulting in a picture similar to that obtained for tissues with high mitotic rates.

A point of additional interest attaches to the relation between volume and DNA content in the granular gland nuclei. This is graphically shown in Figure 5 which includes the data from both tail and back regions. Each plot shows the average

DNA content for all nuclei of the same size. It is seen that nuclear volumes increase regularly with DNA content. It may be observed, incidentally, that DNA values intermediate between class peaks are associated with intermediate nuclear volumes, which suggests that the error in photometry cannot be great.

Leuchtenberger and Schrader (1952) found that in the salivary gland of the snail *Helix* DNA content likewise varies directly with nuclear size, though both decrease with the production of cytoplasmic secretion. No loss of DNA accompanying secretory activity has been found in the granular glands. In this respect the granular glands resemble the pharyngeal and thoracic glands of honeybees, in

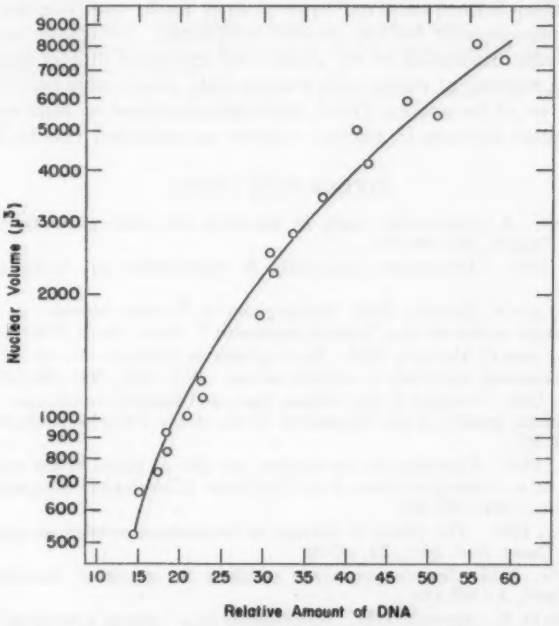

FIGURE 5. Relation between volume and DNA content in the granular gland nuclei of the salamander, *Taricha granulosa*.

which Merriam and Ris (1954) noted a positive correlation between degree of ploidy and secretory activity.

SUMMARY

1. Relative DNA content was determined photometrically for individual Feulgen-stained parenchymal nuclei of the liver, kidney, pancreas and granular glands of the salamander *Taricha* (*Triturus*) *granulosa granulosa* Skilton. Graphs of these values indicated the presence of nuclear classes falling into polyploid ratios.

2. A total of 920 liver nuclei were measured in one animal and 644 in another. A vast majority of the DNA values fell into the first class, representing the

diploid condition. Only 2.6 per cent and 2.2 per cent, respectively, of the total number of nuclei constituted class II, which included values roughly double those of class I. No higher values were found.

3. In the kidney tubules, 136 and 129 nuclei were measured. All could be included in class I.

4. In the pancreas 685 nuclei were measured in one animal, 944 in another. Only 5.2 per cent and 1.6 per cent, respectively, of the total number of nuclei fell into the range of tetraploid values. No higher values occurred.

5. Degree and frequency of polyploidy was marked in the granular glands of the integument. Diploid, tetraploid, and octoploid values were obtained, and presumably higher values occurred in very large nuclei too irregular in shape for photometric measurement by the method employed. Polyploid nuclei increased in frequency with maturation of the glands and exceeded diploid types in number. A considerable number of nuclei with intermediate values suggested the occurrence of aneuploidy or of incomplete DNA replication incident to high endomitotic activity. A regular increase in nuclear volume accompanied rise in DNA content.

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